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Obesity is a growing health concern in the United States and worldwide. The chronic, low grade inflammation associated with increased white adipose tissue mass has been linked to chronic metabolic disorders such as insulin resistance, hypertension, and hyperlipidemia. Consumption of high fat diets leads to intestinal disorders such as microbial dysbiosis and gut barrier dysfunction that can adversely impact systemic metabolism. One potential dietary strategy to alleviate the high fat-induced chronic inflammation is increased consumption of fruits and vegetables rich in polyphenols due to their antioxidant and anti-inflammatory properties. Notably, several studies have demonstrated that supplementation with grape products or anthocyanins found in grapes reduced inflammation systemically and increased the abundance of beneficial bacteria such as *Lactobacillus*, and *Bifidobacterium* in culture. However, the beneficial properties of whole grape powder and one or more of its fractions on intestinal inflammation, microbial populations, and barrier function in mice fed an American type diet rich in saturated fat are unclear.

Therefore, the specific aims of this research were to; (i) determine the impact of consuming California table grapes on intestinal health in mice fed an American type diet rich in one type of saturated fat (Aim 1), and (ii) identify a key fraction (i.e., extractable polyphenol (EP) or non-extractable polyphenol (NEP) fractions) of California table grapes that improves markers of intestinal inflammation in mice fed an American type diet rich in four types of saturated fats (Aim 2). In Aim 1, consumption of the powder grape diet; (i) reduced body fat percentage, the total weight of all four fat depots, and inguinal fat depot weight, (ii) increased localization of a tight junction protein linked to improved barrier function, (iii) reduced the abundance of a deleterious sulfidogenic bacteria, and (iv) increased the abundance of beneficial bacteria (e.g., *Akkermansia*

muciniphila, *Bifidobacterium*, and *Lactobacillus*), compared to high fat controls. However, the high fat diet did not significantly increase the abundance of inflammatory markers in the intestine nor did the powdered grapes significantly decrease their abundance. Taken together, these data acquired in Aim 1 demonstrate that whole powdered California table grapes improved a marker of gut barrier function and a metabolic profile that was positively correlated with changes in microbiota in mice fed a butter-rich diet. In Aim 2, the polyphenol-rich EP fraction alone or in combination with NEP (EP+NEP), but not unfractionated powdered grapes; (i) decreased body fat percentage, body fat depot weights, and liver triglyceride levels, (ii) improved insulin sensitivity and glucose disposal, (iii) decreased the mRNA levels of several inflammatory genes in WAT, and (iv) decreased the expression of the proinflammatory gene Cluster of differentiation 68 in the colon. Taken together, these data demonstrate that the; (i) potential health benefits of consuming grape powder are dependent on the type and amount of fat in the diet, and (ii) extraction of polyphenols from powdered grapes results in improved metabolic profile and decreased systemic inflammation in conjunction with consuming a rich in saturated fats. Collectively, these findings are expected to contribute insight for the development of dietary strategies using table grapes for decreasing obesity and some of its metabolic complications, possibly by altering populations of gut microbes. However, clinical trials are needed to determine the extent to which these findings are applicable to humans.

THE IMPACT OF CALIFORNIA TABLE GRAPES ON INTESTINAL INFLAMMATION
AND MICROBIOTA IN MICE FED AN AMERICAN TYPE DIET

by

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CHAPTER I

INTRODUCTION

Overview

Obesity is a growing health issue, affecting approximately 11% of the world's population and 36% of Americans [1, 2]. Sedentary lifestyle and excessive calorie intake of foods and beverages high in sugars or fats, especially saturated fat, are believed to be the main environmental causes for this disease state [3]. Obesity is of particular concern, because of its comorbidities including type 2 diabetes, hyperlipidemia, and hypertension.

Foods containing indigestible carbohydrates commonly referred to as fiber and bioactive components such as phytochemicals have been shown to reduce some of the deleterious conditions associated with obesity. For example, grapes contain numerous phytochemicals including flavonols, anthocyanins, flavan-3-ols, and hydroxycinnamic acids which have been reported to have antioxidant and anti-inflammatory properties that may alleviate oxidative stress and insulin resistance associated with chronic, low grade inflammation [4, 5]. In addition to phytochemicals, grapes contain fiber, an indigestible food component that has been shown to increase butyrate-producing bacteria, which in turn improves intestinal health by enhancing barrier function [reviewed in 6]. However, little is known about the potential benefits of grapes on intestinal microbiota abundance, inflammatory status, or barrier function in mice consuming an American type diet rich in saturated fat.

Central Hypothesis and Specific Aims

The long term goal of our research group is to develop innovative dietary strategies designed to control chronic, low-grade inflammation commonly associated with diet-induced obesity using California table grapes. The central hypothesis for my thesis is that intestinal inflammation and impaired gut barrier function associated with diets high in saturated fats will be attenuated by dietary intervention with California table grapes through the modification of intestinal microbiota and inflammatory status as shown in the working model (**Fig. 1.1**). Our group is well prepared to carry out this research due to our experience; (i) in laboratory animal husbandry protocols, (ii) live animal testing procedures, and (iii) tissue analyses including RNA isolation, cDNA synthesis, qPCR, immunoblotting, and enzyme assays, thereby ensuring minimization of errors in data collection. Our collaborators at the University of Illinois are Dr. H. Rex Gaskins and graduate assistant Patricia Wolfe, experts in microbiota analyses and culturing probiotics. Our collaborators at the North Carolina Research Campus in Kannapolis are Drs. Mary Ann Lila and Mary Grace, experts in fractionating and identifying fruit polyphenols, and Drs. Zhan Zhou and Wei Zong, experts in assessing gut barrier function. Our collaborators at the University of Chicago are Drs. Kristina Martinez, Eugene Chang, and Chase Cockrell, experts in microbiota and short chain fatty acid (SCFA) analyses.

To test my hypothesis, I will pursue the following two specific aims:

- Aim 1.* Determine the impact of consuming California table grapes on intestinal health in mice fed an American type diet rich in one type of saturated fat; and
- Aim 2.* Identify a key fraction of California table grapes that improves markers of intestinal inflammation in mice fed an American type diet rich in four types of saturated fats.

Based on my review of the current literature, the ability of California table grapes, the most abundantly-grown table grapes in the U.S., to suppress intestinal inflammation and improve barrier function in mice fed an American type diet has not yet been examined. Furthermore, the bioactive fraction in table grapes responsible for these potential gut health-promoting actions and their impact on goblet cell function is unknown, and the impact of these grapes on gut microbiota has not been published. Thus, completion of my aims will lead to the following anticipated outcomes; (i) powdered grapes will attenuate gut inflammation in high fat fed mice, as well as increase the number of butyrate-producing bacteria while reducing the number of sulfidogenic bacteria, and (ii) a polyphenol-rich, bioactive fraction (i.e., containing extractable polyphenols) in table grapes will be identified that reduces adiposity and improves glucose tolerance in association with reducing intestinal inflammation. These proposed improvements will be associated with modifications in mucosal microbiota (i.e., decreasing sulfidogenic bacteria and increasing butyrate producing bacteria (i.e., *Bifidobacterium*) or *Akkermansia muciniphila*, (a health-promoting microbe), and upregulation of G-protein receptors (i.e., GPR-41,-43, or -119) associated with improved metabolic profiles.

Completion of this research will further our understanding of how California table grapes act as prebiotics that improve gut health and subsequent metabolic complications associated with consuming a diet rich in saturated fat. Such knowledge will inform the public about the gut health-promoting properties of California table grapes; a nutritionally and economically important agricultural products in the U.S.

Significance

Diets high in fats and calories lead to obesity and are associated with chronic, low-grade intestinal inflammation and alterations in the intestinal microbiota [reviewed in 7]. My research will demonstrate that powdered California table grapes will attenuate the deleterious changes

associated with consuming diets rich in saturated fatty acids. The research proposed in this study is significant, because application of the results and new knowledge gained are expected to lead to new dietary approaches using California table grape products to reduce intestinal inflammation with improvements in intestinal and systemic health. These proposed outcomes have the potential to reduce health care costs and loss of productivity associated with obesity and its comorbidities.

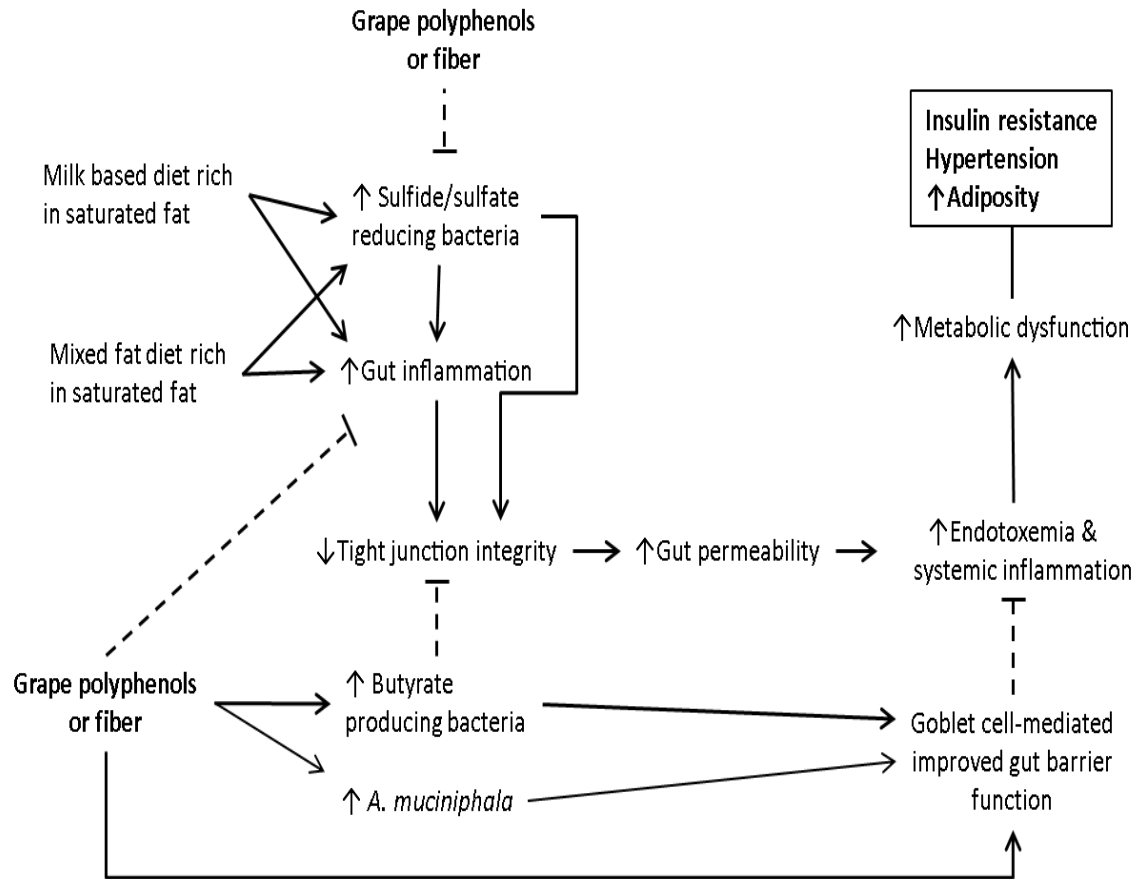


Figure 1.1. Working Model. Powdered grapes attenuate inflammatory signaling, endotoxemia, and metabolic dysfunction in mice consuming an American diet rich in saturated fats by; (i) increasing butyrate-producing bacteria and *Akkermansia muciniphila* (*A. muciniphila*) which enhance goblet cell secretion of mucin and barrier protein synthesis in colonocytes, (ii) reducing intestinal inflammation which will prevent gut permeability, (iii) attenuating the growth of sulfide/sulfate reducing bacteria which produce the cytotoxic gas hydrogen sulfide that increases gut inflammation and permeability.

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CHAPTER II

REVIEW OF LITERATURE

Obesity and the Consequences of Chronic Increases of Inflammatory Genes

An estimated 1.4 billion people over the age of 20 are over-weight and an additional 500 million are considered obese worldwide [1]. As of 2010, an estimated 69% of the United States' adult population was considered overweight or obese (36%) [2]. Consumption of high calorie diets and sedentary lifestyles are two major causes of obesity. Obesity or excess body fat is defined as having a body mass index (BMI) >30 [3]. The health risks of obesity include high blood pressure, type 2 diabetes, and hyperlipidemia, which have been referred to as the metabolic syndrome [3].

An important linkage between obesity and its associated health risks is chronic, low-grade inflammation. One mediator of inflammation is tumor necrosis factor alpha (TNF- α), which has been shown to cause insulin resistance in muscle and white adipose tissue (WAT) [4, 5]. TNF- α is a cytokine produced by immune cells and adipocytes that increases insulin resistance via inhibition of insulin signaling cascades needed for glucose uptake and utilization [6]. Notably, TNF- α expression is elevated in WAT and muscle of human and animal obesity models [7, 8]. Consistent with these data, TNF- α knockout mice fed high fat diets did not develop insulin resistance [9]. Thus, TNF- α is a pro-inflammatory cytokine associated with increased WAT that contributes to some of the health risks associated with obesity.

Obesity is typically accompanied by increased macrophage recruitment within WAT. This recruitment not only increases the secretion of cytokines, inducing further inflammation, but is

also involved in tissue remodeling [10]. As adipocytes increase in size in order to compensate for increased triglyceride (TG) synthesis from excessive glucose and fatty acid uptake, they become abnormally large, overstressing the collagen matrix and vasculature of the tissue [11]. These changes result in decreased angiogenesis, hypoxia, and adipocyte apoptosis in WAT, leading to the macrophage recruitment designed to clean up necrotic adipocytes in WAT.

Increased gene expression and secretion of interleukin-6 (IL-6) and monocyte chemoattractant protein (MCP-1) are further examples of pro-inflammatory cytokines released by expanding adipocytes [12]. The increase in IL-6 results in an increase in c-reactive protein (CRP), which is a cytotoxic, acute phase protein. MCP-1 increases adipocyte inflammation through recruitment of classically-activated macrophages (i.e., M1) which cause the release more pro-inflammatory cytokines including CRP, cluster of differentiation 11c (CD11c), and enzyme inducible nitric oxide synthase (iNOS).

The increased expression of inflammatory peptides or proteins is initiated by an inflammatory kinase cascade. Inflammatory mediators such as inflammatory cytokines and lipids, endoplasmic reticulum (ER) stress, and reactive oxygen species (ROS) activate mitogen-activated protein kinases (MAPK)s, c-Jun N-terminal kinase (JNK) and I κ B kinase (IKK) [13]. JNK and IKK modulate a phosphorylation signaling cascade resulting in the activation of transcription factors activation protein-1 (AP-1, aka cJun) and nuclear factor-kappa B (NF- κ B), respectively.

Activation of these transcription factors leads to an increase in the expression of the inflammatory cytokines, exacerbating the inflammatory response. Hyperglycemia and hyperlipidemia due to excess energy consumption increases mitochondrial metabolism leading to an increase in ROS production. ER protein synthesis is also increased in response to mitochondrial need and metabolic stress, resulting in ER stress. Increasing JNK and IKK may also inhibit insulin receptor substrate activation via serine phosphorylation, resulting in insulin resistance [14].

Other tissues are also affected by the excessive caloric intake. For example, skeletal and cardiac muscle tissue can experience an increase in free fatty acids (FFA) uptake when blood levels are elevated, resulting in ectopic deposition in these tissues [reviewed in 15]. As in WAT, this leads to an overload of the capacity of ER to metabolize FFAs, leading to an increase in ceramide and diacylglycerol and an inflammatory cascade similar to WAT, resulting in insulin resistance. Increased incorporation of lipids and cyto-chemokine signaling in endothelial cells also leads to increased macrophage accumulation of lipids, resulting in the development of foam cells, compromised blood flow, and cardiovascular disease [16]. Lastly, ectopic accumulation of lipids in pancreas can impair insulin secretion, thereby reducing insulin-stimulated glucose uptake and utilization.

Relationship between Systemic and Intestinal Inflammation

Although systemic inflammation has been a primary focus in research involving obesity, the effects of increased WAT on intestinal inflammation, and vice versa, have more recently been recognized. Along with instigating chronic, low grade inflammation systemically, diets high in fats also impact intestine health. However, the relationship between WAT and intestinal inflammation due to diet-induced obesity is unclear; i.e., does obesity cause WAT inflammation, which in turn, causes intestinal inflammation and barrier dysfunction, or does intestinal dysbiosis and inflammation due to high fat feeding cause endotoxemia, increased energy harvest, WAT expansion, and inflammation? These questions are currently being debated. Whereas some studies indicate that obesity triggers the release of inflammatory signals from WAT that influence the intestinal tract [17], others indicated that high fat diets trigger intestinal inflammation or endotoxemia, that causes systemic inflammation, possibly due to intestinal barrier dysfunction. For example, activation of the Toll-like receptor 4 (TLR4)/NF- κ B signaling pathway by saturated

fatty acids or lipopolysaccharide (LPS) increases TNF- α and IL-6 expression in intestinal tissue of high fat fed mice [18]. High fat diets have also been shown to modulate the ratio of immune cells produced by intestinal lymphocytes [19]. In particular, mice fed a high fat diet were more susceptible to dextran sulfate sodium (DSS) induced colitis due to an increase of non-CD1d-restricted natural killer T (NK T) cells as opposed to regular NK T cells in the colon. Non-CD1d-restricted NK T cells produce inflammatory cytokines similar to those expressed in adipocytes leading to an increased susceptibility of colon mucosa to a DSS insult, whereas regulatory T cells would protect against such an insult reducing the occurrence of colitis.

Gut Barrier Function and the Role of Gut Microbiota

The landscape of the gastrointestinal (GI) tract is one of constant change. Diet composition and microbiota-sensing proteins like TLR4 signal not only for modulation of gene and protein expression, but also for the differentiation of epithelial cells. Crypt cells utilize these signals to differentiate epithelial stem cells into enterocytes, goblet cells, enteroendocrine cells, or antimicrobial peptide secreting cells (e.g., paneth cells), based on the need for increased absorption, physical barrier, chemoreceptors, or microbial regulation, respectively [reviewed in 20]. Despite the constant turnover of epithelial cells, which allows for continuous migration of cell types to areas of need, all of the cell types display tight junction proteins such as claudin-1, occludin-1, or zonula occludens (ZO)-1, forming an effective barrier between cells [21]. Just as the adaptability of epithelial cells and tight junctions between them are meant to be protective mechanisms against the entry of pathogens and deleterious compounds (i.e., bacterial LPS, DNA, or peptidoglycan) into the bloodstream resulting in endotoxemia), so are the secretions of goblet cells.

Goblet cells form and secrete mucin glycoproteins and mucins, which create a bilayer lining the intestine [22]. The inner layer closest to the enterocytes prevents the passage of pathogens into the enterocyte. The outer layer is more loosely connected creating a homeostatic environment suitable for bacterial attachment and growth [23]. The benefits of mucins have been demonstrated in the development of ulcerative colitis in mucin 2 (MUC2)-depleted mice [24].

Similar to constant epithelial cell turnover, the composition of the microbiota fluidly changes depending on the available dietary substrates consumed and changes in pH resulting from the level of acidic byproducts generated [reviewed in 25]. Thus, the symbiotic relationship between the endothelial cells and microbes can be disrupted by adverse environmental changes such as high fat diets (**Table 2.1**). Dysfunction of this system is represented by increased plasma, urine, and fecal endotoxin levels, as well as markers of inflammation in the gut including the macrophage recruiter monocyte chemoattractant protein (MCP-1) and TLR4. However, dietary changes to the gut microbiome may be independent of such dysfunctions or obesity. For example, Sprague Dawley rats fed a high fat diet were categorized as obesity-prone or obesity-resistant. Regardless of their designation, all high-fat-fed rats exhibited a reduction in total microbial density with an increase in *Bacteroidales* and *Clostridiales*. However, obesity-prone rats exhibited an increase in intestinal markers of inflammation and reduction of gut barrier function leading to increased levels of plasma LPS. Thus, microbial alterations were independent of increased inflammation and obesity. Of greater concern is the development of endotoxicity.

Endotoxicity is represented by an increase in serum levels of lipopolysaccharide (LPS), a cell wall component of gram negative bacteria, demonstrating microbial inflammation and compromised barrier function. This was demonstrated in research involving TLR4-deficient and normal mice [27]. Both normal and TLR4-deficient mice fed a high fat diet showed alterations to the gut microbiome through an increase in the ratio of *Firmicutes* to *Bacteroidetes*, primarily

through an increase in *Enterobacteriaceae*, a gram-negative bacterial family, which coincided with an increase in intestinal inflammation and impairment of gut barrier function compared to low fat controls [27]. The high-fat-fed mice also had increased systemic inflammatory markers including those related to endotoxicity compared to low-fat-fed mice. The TLR4-deficient mice had a similar increase in body weight compared to the wild type, high-fat-fed mice. However, epididymal tissue weight, and plasma endotoxin levels were significantly decreased in conjunction with the TLR deficiency, demonstrating a correlation between TLR4-mediated inflammation and increased adiposity and endotoxemia. Thus, the impact of diet on gut microbiota has a major effect on systemic inflammation.

Association of Gut Microbiota with Chronic Disease States

Research has attempted to determine potential mechanisms by which microbiota modulate intestinal inflammation (Table 2.1). Primarily, there are indications that a correlation exists between CD4⁺ T, Th1, and Th17 cell response to antigens produced by bacteria and chronic intestinal inflammation associated with inflammatory bowel disease (IBD) [reviewed in 28]. These immune response cells are activated by specific stimuli associated with commensal bacteria and their abundance may be increased in healthy GI tracts.

The development of diseases associated with chronic inflammation may be the result of alterations in commensal populations as opposed to introduction of a pathogenic strain. Recent research using ulcerative colitis mouse models, which developed intestinal dysbiosis, demonstrated modular increases in *Enterobacteriaceae*, *Klebsiella pneumonia*, and *Proteus mirabilis*, that induced colitis when transferred to wild-type mice [29, 30]. IL-10 deficient gnotobiotic mice monoassociated with nonpathogenic strains of either *Escherichia faecalis* or *E. coli* induced colitis, whereas similar monoassociation with wild type gnotobiotic mice did not.

Interestingly, alterations in the colitis phenotypes were observed to be dependent on the strain. *E. faecalis* induced slower onset with predominant colitis in the colon whereas *E. coli* induced a quicker onset predominating in the cecum [31, 32].

Such results may give some indication as to the association between location of site-specific IBD and certain bacterial blooms. Patients with Crohn's disease in particular have notably reduced microbial diversity, yet an increase in epithelial surface colonization and infiltration [33]. Indeed, certain bacteria dominate these colonizations depending on the sight of inflammation as demonstrated by the decrease in *Faecalibacterium* and *Rosburia* and increase in *Enterobacteriaceae* and *Ruminococcus gnavus* populations associated with Ileal Crohn's Disease. In another study biopsies of intestinal tissue of patients with ulcerative colitis and Crohn's disease compared to healthy controls demonstrated that *Proteobacteria* and *Bacteroidetes* were significantly increased by Crohn's disease while *Clostridia* were decreased compared to healthy and ulcerative colitis patients [34]. These increased bacterial populations associated with IBD are primarily commensal bacteria that normally do not produce inflammation. Thus, it is suspected that a genetic component is also involved. Indeed, the majority of genetic risk factors associated with IBD encode for proteins involved in sensing the microbial environment (e.g., nucleotide-binding oligomerization domain containing 2), host immune response elements (e.g., the IL-12-IL-23R pathway), and gut barrier function [reviewed in 35].

Like IBD, non-alcohol fatty liver disease may be caused by endotoxicity resulting from impaired gut barrier function. A correlation has been found between obesity due to high-fat intake, alterations in gut microbiota, and associated intestinal permeability and non-alcohol fatty liver disease [reviewed in 36]. Although non-alcohol fatty liver is associated with obesity, not all obese individuals develop this condition. This may be due, in part, to individual variability of the host microbiome. Indeed, evidence in animal and human studies suggests that choline dependent

bacteria may have a great impact on this disease state [37, 38]. In both cases, the increase of choline dependent bacteria such as *Gammaproteobacteria* and *Erysipelotrichi*, resulted in conversion of choline to methylamines creating a choline depleted environment. In such cases, liver steatosis increased, providing further evidence to the causal link between microbiota and liver disease.

As inflammation progresses, it results in nonalcoholic steatohepatitis and liver cirrhosis. As such, the level of cirrhosis has been shown to be correlated with increased levels of LPS, resulting in activation of nitric oxide modulated enzymes [reviewed in 36]. Furthermore, cirrhosis is evidenced by a depressed endothelial defense system as a result of bacterial translocation.

Impact of Diet on Gut Microbiota and the Potential Influence on Host Health

Several studies have linked high-fat feeding to remodeling of microbial populations in the GI tract (Table 2.1). The most common outcome of these high-fat-feeding studies is an increase in *Firmicute/Bacteroidete* ratio in obese subjects [39-41]. This led to an increase in energy harvesting (e.g., increase in short chain fatty acid (SCFA) absorption) and a corresponding increase in adiposity. Germ-free mice inoculated with microbes from obese mice had significantly increased fat accumulation with no dietary modification; further supporting the concept that changes in gut microbes influences the development of obesity [39]. An acute inflammatory response due to the increase in *Firmicute/Bacteroidete* ratio has also be correlated with a significant increase in the levels of CRP and fecal calprotectin, two proteins secreted in response to pathogenic inflammation [40].

Other indicators of microbiota influence on inflammation in C57BL/6J mice fed high fat, low carbohydrate diets include lower expression levels of intestinal tight junction proteins and increased markers of WAT inflammation and body fat compared to low-fat-fed controls [41-43].

Furthermore, several research studies have demonstrated that changing the diet from high fat to low fat has a quick, positive effect on the composition of microbiota and subsequent intestinal inflammation [44, 45]. This demonstrates not only the adaptability of the microbiota to dietary changes, but also that of epithelial integrity and immune response systems. Indeed, dietary changes can induce complete changes in the gut microbiota in as little as 24 hours after dietary change [46].

Emerging research has linked products of microbiota metabolism as a primary cause for changes in intestinal integrity and inflammation. Such metabolic products are influenced by dietary components. For example, hydrogen sulfide is a byproduct of some intestinal microbes such as *Bilophila wadsworthia* and *Desulfovibrionaceae* that has emerged as a major deleterious compound associated with high-fat-feeding [47, 48]. Increased hydrogen sulfide resulted in decreased gut barrier function, increased immune response, and reduction of gut-barrier protecting bacteria, like *Bifidobacterium* [48]. Indeed, multiple studies have shown a positive correlation between increased hydrogen sulfide and development of ulcerative colitis, gut inflammation, IBD, and colon cancer [47-49]. This is due, in part, to the cytotoxic nature of hydrogen sulfide on colonocytes, leading to increased turnover, along with reductions in butyrate oxidation [50-52].

Milk fat, but not lard or polyunsaturated fat, increased *Bilophila wadsworthia* populations, which was associated with increased incidence of colitis [48]. This was primarily due to higher hepatic taurocholic acid production, a preferred substrate for sulfidogenic bacteria. Thus, the type of fat has a marked effect on gut microbes and their metabolic products, which in turn, impact gut health. This is important to consider when designing dietary strategies to attenuate intestinal and systemic inflammation.

Dietary compounds that pass through the upper GI unabsorbed become potential substrates for gut microbes. Resistant carbohydrates are a class of potential substrates for fermentation that impact gut microbiota and their metabolic products (**Fig. 2.1**). Resistant starches, non-starch polysaccharides, and oligosaccharides make up what is commonly referred to as fiber. The resultant fermentation of these substrates leads to production of volatile SCFAs, CO₂, H₂, and formate [53]. The SCFAs most abundantly produced are acetate, propionate, and butyrate. The acidic nature of these products reduces intestinal pH throughout the lower GI, potentially preventing the growth of pathogenic bacteria (i.e., *Enterobacteriaceae*) [54, 55]. This effect on pH may also be a determining factor on which class of fermenters predominate. At more neutral pH (6.5), acetate producers predominate whereas in a more acidic environment (pH 5.5), butyrate producers predominate [56]. Indigestible oligosaccharides facilitate a lower pH allowing butyrate producers to compete for substrates more efficiently than acetate or propionate producers that have slower growth [57].

The majority of SCFAs absorbed by gut epithelial cells are metabolized and the rest enter the hepatic-portal circulation. For example, butyrate is the primary energy source for endothelial cells, whereas propionate is sent to the liver where it is used as a substrate for gluconeogenesis [58]. Aside from being potentially the preferred energy source of enterocytes, butyrate also increases goblet cell gene expression, including MUC2 [59], and inhibits NF-κB signaling thereby reducing inflammation [60, 61]. Acetate can be used by the liver and WAT as a substrate for triglyceride (TG) or cholesterol biosynthesis. Lactate is another fermentation product that is primarily utilized as a substrate for other bacteria [62, 63], or converted to other SCFAs. Increased butyrate production has multiple beneficial effects on intestinal integrity (i.e., increased gut barrier protein synthesis and mucin secretion that improve gut barrier function, reduce endothelial cytokine production, and regulate intestinal inflammation).

Polyphenols are naturally occurring phytochemicals in plants that have been shown to enhance a number of biological activities, in particular those involved in the suppression of inflammation and oxidative stress and carcinogenesis [reviewed in 64]. Polyphenols are powerful antioxidants, having been shown reduce levels of ROS, reactive nitrogen species, hydrogen peroxide, and nitric oxide. Reducing pro-oxidants, in turn, decreases pro-inflammatory signaling pathways [64]. In addition, polyphenols exhibit apoptosis signaling properties that have been linked to cancer prevention. In vitro studies using breast and pancreatic cancer cell models have shown that resveratrol and epigallocatechin gallate from red wine promoted apoptosis through several signaling pathways, thus reducing the proliferation of the cancer cells in culture [65, 66]. Polyphenols have also been shown to inhibit inflammatory processes and microbial infiltration into intestinal cells. For example, pomegranate extract inhibited NF- κ B activity in human epithelial colorectal adenocarcinoma (Caco2) cells, reducing the level of secreted cytokines [67]. Similarly, geletin prevented enteric bacteria from entering Caco2 and HT-29 cells, a human colorectal adenocarcinoma cell line [68]. Although polyphenol absorption can be detected almost immediately and peak concentrations occur as quickly as 2 hours post-ingestion [69], the relative abundance in circulation is very low compared to dietary intake and urinary excretion levels [reviewed in 70]. However, known metabolites of polyphenols can be detected in greater concentrations systemically and in feces with different kinetic profiles, indicating that bioaccessibility and bioavailability are complex processes involving multiple stages of liberation, primarily by microbial enzymes prior to their absorption, distribution, and excretion [71].

Over 90% of dietary polyphenols are not absorbed in the upper GI and thus persist in the lower GI tract [reviewed in 72]. This is primarily the result of low concentrations of polyphenols within the food and enzymes necessary to metabolize polyphenols into more readily absorbable forms (**Fig. 2.2**). Naturally occurring polyphenols require deconjugation in order to diffuse into the

enterocyte [72]. The brush border of the small intestine contains membrane bound β -glucosidases which facilitate the process for hydrolyzing glucuronated polyphenols into absorbable aglycones [73]. Once within the enterocyte, the aglycone will either undergo Phase I (e.g., reduction, oxidation, or hydrolysis) and II (e.g., conjugation) metabolism immediately, converting them into methyl-esters, glucuronides, and sulfates primarily, or be transferred to the liver to undergo similar metabolism [reviewed in 73]. Conjugating aglycones reduces their potential microbial toxicity, while also making them easier to transport as biotransformed polyphenols. Subsequently, they may enter the systemic circulation where they may interact with other tissues, be excreted in the urine, or become bound to bile acids and redistributed back into the intestine following release from the gall bladder [reviewed in 71]. Those polyphenols that are easily absorbed may play some role in the observed health benefits; however, their health benefits are more likely the result of their interaction with gut microbiota.

Because the majority of polyphenols persist to the distal small intestine and colon, it is believed that their interactions with the gut microbiota play a greater role in health promotion and disease prevention (Fig. 2.2) [71]. Polyphenols exhibit similar effects on the luminal environment as previously reported with fiber, including reducing pH, thereby impacting the growth of some bacteria while also being a substrate for bacterial fermentation [reviewed in 74]. Research has demonstrated that bacteria, primarily gram-negative anaerobes, are able to metabolize most polyphenols regardless of complexity or conjugation through the use of a variety of enzymes (e.g., esterase, glucosidase, glycosidase, dehydroxylation, decarboxylation, demethylation, and ring fission activity) into generalizable metabolites (e.g., acetate, propionate, butyrate, gallic acid, hippuric acid, ferulic acid) [75-78]. Aside from their influence on pH, polyphenols also exhibit other antibacterial properties (i.e., inhibit quorum sensing [79], disrupt lipid membrane integrity [80] and DNA polymerase activity [81], and alter protein synthesis priority from metabolism to

protection [82]). As such, polyphenol metabolism to non-toxic byproducts may be a defense mechanism. Nevertheless, the presence of polyphenols and their metabolites may have beneficial effects on the gut microbiome and host health.

The ability of some microbes to metabolize polyphenols and antibacterial properties exerted on others demonstrate their prebiotic potential (Fig. 2.2). Caco2 cells in a mix batch culture media treated with quercetin and naringen reduced the adhesion of *S. aureus* while improving the growth of *Lactobacillus* [83]. Pomegranate extract supplemented in high-fat-fed Balb/c mice increased *Bifidobacterium* levels in the cecum, which correlated with decreased inflammation in the colon and visceral adipose tissue [84]. Similarly malvindicin-3-glucosides, gallic acid, and a mixture of anthocyanins enhanced the growth of *Bifidobacterium* and *Lactobacillus-Enterococcus* bacteria [85]. Indeed human males given a proanthocyanin-rich extract had a dramatic shift in fecal microbial populations from *Bacterioides*, *Clostridium*, and *Propionibacterium* phyla to *Bacterioides*, *Lactobacillus*, and *Bifidobacterium* predominance [86]. Taken together these studies strengthen the concept that polyphenol consumption effectively reduces deleterious bacteria while promoting beneficial bacteria. In addition to the observed prebiotic effects, microbial metabolism of polyphenols has a positive influence on host health.

As previously mentioned in regards to fiber, polyphenol metabolism may produce byproducts beneficial not only to microbiota homeostasis, but also to host health. Metabolites tested in vitro have been shown to consistently inhibit iNOS, cyclooxygenase-2, IL-1 β , IL-6, TNF- α , as well as preform as antioxidant and antiproliferative agents [reviewed in 71]. The ring cleavage of flavonoids into SCFAs similar to fermentation of fiber, has the same beneficial effects on energy intake, metabolism regulation, and improvements to epithelial health and integrity [87]. Other metabolites such as gallic acid, hippuric acid and ferulic acid are less likely to be utilized as a substrate by bacteria and thus are more readily absorbed into the systemic circulation. In vitro

testing of the degradation of gallic acid compared to anthocyanins in a model representing colonic bacterial cultures showed that anthocyanins were completely degraded within 5 hours while gallic acid remained in measureable concentrations for 24 hours [85]. Human subjects given an anthocyanin supplement had 17 distinguishable metabolites recognized within their serum that have a wide range of kinetic profiles, demonstrating that metabolites of polyphenols can exist long after the initial peak observed when measuring the levels of the polyphenol itself [88]. Taken together, these results as well as previously mentioned results involving polyphenols and fiber suggest that foods particularly berry fruits including grapes, containing bioactive compounds that can have a positive impact on intestinal and systemic health through their interactions and influence on the intestinal microbiome.

Anti-inflammatory Properties of Grapes

Grapes and their byproducts are commonly consumed throughout the world. Grapes contain nutrients such as water, carbohydrates, fats, vitamins, minerals, and fiber as well as bioactive phytochemicals, particularly polyphenols [reviewed in 89]. The phytochemicals and the fiber components of grapes have been hypothesized to attenuate diet induced, chronic inflammation and pro-oxidants [reviewed in 89]. The phytochemicals in grapes include flavonoids, (pro) anthocyanidins/anthocyanins, and stilbenes [89]. Grapes also contain fiber in the form of cellulose, pectin, and various fructans (e.g., fructo-oligosaccharides, disaccharides, and polysaccharides), which are the main indigestible carbohydrate component of grapes [90, 91].

Anthocyanins are the most abundant polyphenol group in grapes and many other berries [reviewed in 90]. In vitro studies have demonstrated that anthocyanins reduce ROS and insulin resistance in adipocytes exposed to hydrogen peroxide or TNF- α [92]. Consumption of anthocyanin-rich tart cherry powder reduced adiposity, WAT NF- κ B activity, and serum TNF- α

and IL-6 levels in high fat fed rats [93]. Mice fed anthocyanin-rich blueberry powder had reduced levels of inflammation markers, oxidative stress, and insulin resistance compared to controls [94]. Flavanols, primarily quercetin, kampferol, rutin, and isorhamnetin, have also been shown in vitro to reduce oxidative stress in multiple cell types exposed to several inflammatory compounds [95-99]. In vitro testing of liver cells exposed to a cytokine mixture demonstrated that quercetin and kaempferol attenuated an increase in ROS and oxidized glutathione, demonstrating their ability to suppress pro-oxidants [95]. Rutin has been shown to reduce inflammatory gene expression in epithelial sepsis cell models [96]. Similar results were seen in mice treated with quercetin; i.e., quercetin treatment in mice fed a high fat diet lowered mRNA levels of pro-inflammatory IL-6, CRP, MCP-1, and acyloxyacyl hydrolase in liver tissue compared to high fat controls [97]. Isorhamnetin, a metabolite of quercetin, reduced LPS- induced oxidative stress in rats [98]. Intestinal colitis was attenuated by concentrated grape juice in Wistar rats, with flavonoids being the proposed facilitators of these beneficial changes in gut health [99].

Potential Prebiotic Properties of Grapes

To date, the prebiotic effects of California table grapes have not been explored. However, studies have been conducted on grape products, extracts, and polyphenols including quercetin, fructo-oligosacharrides, and grape juice. Inoculation of *Lactobacillus acidophilus* and *plantarum*, two probiotic bacteria, with quercetin plus fructo-oligosacharrides increased their growth compared to controls [100]. Fructo-oligosacharride has also been shown to enhance the growth of butyrate-producing bacteria from *Firmicute* and *Bifidobacterium* families [101]. Several grape juice varieties have also demonstrated to have prebiotic effects by increasing the growth of *L. acidophilus* and *L. delbruekii*, two probiotic bacteria, while attenuating growth of *E. coli* in vivo.

[102]. However, the impact of California table grapes on intestinal health and its association with systemic health in diet-induced obesity has not been reported in the literature.

Grape polyphenols may have a major impact on the microflora of the large intestine. Red wine grape polyphenols given to humans for 4 weeks significantly increased the number of *Enterococcus*, *Prevotella*, *Bacteroidetes*, and *Bifidobacterium* bacteria which were positively correlated to improved blood pressure, and serum concentrations of triglycerides, total cholesterol, and CRP [103]. F344 rats treated with wine polyphenols also had increased numbers of beneficial bacteria namely *Bacteriodes*, *Lactobacillus*, and *Bifidobacterium* [104]. Grape seed extract and wild blueberry supplementation increased *Bifidobacterium* levels [105, 106]. Further evidence of the prebiotic effects of grape consumption was demonstrated in rats given grape pomace juice, which increased fecal counts of *Lactobacillus* and *Bifidobacterium* and consequently resulted in an increase in the concentration of primary bile acids, cholesterol, and cholesterol metabolites while decreasing the concentration of secondary bile acids [107]. This indicates that alteration of the microflora by grape polyphenols may inhibit cholesterol absorption, thereby lowering circulating cholesterol levels. Although these studies were conducted in humans and rats with wine grape, grape seed extract, and blueberries, there is reason to believe that similar results should be demonstrated in mice fed California table grapes.

Summary and Gaps in the Literature

The rise in obesity is positively correlated with cardiovascular disease, type 2 diabetes, and hypertension via induction of chronic, low-grade inflammation. Overconsumption of calories relative to energy expenditure results in WAT expansion, hypoxia, and adipocyte death with subsequent production of inflammatory cytokines, chemokines, and adipokines. It also causes ectopic deposition of lipids in pancreas, liver, and muscle that further contribute to ER stress,

insulin resistance, hyperlipidemia, and inflammation. These metabolic outcomes of obesity are associated with macrophage recruitment, which further exasperates inflammatory signaling. Similarly, diet-induced obesity has been linked to intestinal inflammation, which has been associated with changes in populations of gut microbes. Such changes increase the production deleterious bacterial products that together reduce gut barrier function. Barrier dysfunction, in turn, allows translocation of inflammatory signals (e.g., bacterial LPS, DNA, and peptidoglycans) into the blood stream leading to endotoxemia and systemic inflammation. One potential strategy to reduce this inflammatory scenario associated with dietary obesity is to consume foods rich in indigestible carbohydrates and polyphenols that enhance intestinal health. Fructans such as fructo-oligosaccharides have been shown to promote enterocyte health by improving the composition of the microbiota (e.g., increasing butyrate producers and *Akkermansia muciniphila* and decreasing sulfidogenic bacteria) [81, 82]. Similarly, grapes and other berries have been shown to have anti-oxidant and anti-inflammatory properties due to their polyphenol abundance. Thus, many of the proposed beneficial systemic effects of consuming grapes may be due to their content of indigestible carbohydrates and polyphenols that positively influence intestinal health.

However, little is known on whether the concentration of fructans or polyphenols in California table grapes is sufficient to alter gut microbes. Furthermore, the impact of table grapes on intestinal inflammation has not been reported in the literature. In addition, the bioactive fraction of grapes responsible for these potential health benefits is not known.

Based on these gaps, I hypothesize that dietary intervention using whole powdered California table grapes will attenuate intestinal inflammation and improve gut barrier function associated with consuming diets high in saturated fat, and that these beneficial effects will be associated with modifications of intestinal microbes. This hypothesis will be tested by pursuing the following two specific aims,

- Aim 1.* Determine the impact of consuming California table grapes on intestinal health in mice fed an American type diet rich in one type of saturated fat; and
- Aim 2.* Identify a key fraction of California table grapes that improves markers of intestinal inflammation in mice fed an American type diet rich in four types of saturated fat.

Table 2.1. Association between Gut Microbiota and Chronic Disease States

Factors	Change in Microbiota	Health Impacts	Associated Chronic Disease
Milk fat diet	<p>↑ Tauracholic acid→↑ <i>DSB</i>→ ↑ H₂S</p> <p>Inhibits <i>Bifidobacteria</i> growth, butyrate production</p>	<p>H₂S- inhibits TJP gene expression↑intestinal permeability</p> <p>↑immune response–TH1 activation releasing IFN-γ and IL-12</p> <p>Cytotoxic–↑Cell turnover</p>	<p>Ulcerative Colitis Inflammatory Bowel Disease Colon Cancer</p>
High fat diet	<p>↑ <i>Firmicutes</i> ↓ <i>Bacteroidetes</i></p>	<p>↑expression of inflammatory markers (CRP, fecal calprotectin, MCP-1, TLR4) in intestine</p> <p>↓TJP expression→↑ intestinal permeability→↑systemic LPS→systemic inflammatory response to endotoxemia</p> <p>LPS induced ↑ in WAT markers of inflammation→ hyperglycemia & insulin resistance</p>	<p>Intestinal inflammation</p> <p>Obesity</p> <p>Diabetes</p>
Gene Mutations (NOD2, Atg16l1, IL-23R)	<p>↓<i>Rosburia</i> ↓<i>Faecalibacterium</i> ↑<i>Enterobacteriaceae</i> ↑<i>Ruminococcus</i> ↑<i>Enterobacteriaceae</i> ↓<i>Clostridia</i></p>	<p>Impaired sensing of microbial environment (NOD2), host immune response (Atg16l1, IL-23R), gut barrier function:</p> <p>↓microbial diversity; ↑epithelial colonization and infiltration</p>	<p>Chron's Disease</p> <p>Ulcerative Colitis</p>
Choline	<p>↑<i>Gammaproteobacteria</i> ↑<i>Erysipelotrichi</i></p>	<p>Choline depleted luminal environment→↓hepatic choline level→↓ VLDL synthesis→ ↑hepatic lipid storage</p> <p>↑bacteria byproduct TMA in plasma converted to TMAO by liver</p> <p>↓epithelial immune response due to bacterial translocation</p>	<p>Non-alcoholic fatty liver disease due to the inhibition of choline storage</p> <p>Atherosclerosis associated with ↑ TMAO in circulation</p>

Desulfogenic Bacteria (DSB), hydrogen sulfide (H₂S), tight junction protein (TJP), interleukin (IL), interferon (IFN), C-reactive protein (CRP), macrophage chemoattractant protein-1 (MCP-1),

toll-like receptor-4 (TLR4), lipopolysaccharide (LPS), white adipose tissue (WAT), nucleotide-binding oligomerization domain containing 2 (NOD2), autophagy related 16-like 1 (Atg16l1), very low density lipoprotein (VLDL), trimethylamine (TMA), TMA-N-oxide (TMAO).

increased nutrient absorption and potentially resulting in adipogenesis, or (9) in the presence of leptin increase satiety thereby reducing nutrient intake. GLP-1 can also (10) stimulate the release of insulin and increase insulin sensitivity resulting in improved glucose uptake in muscle. GLP-2 improves intestinal immunity through (11) increasing gut barrier function and L cell number and improving microbial regulation. Individually, acetate (12) increases triglyceride and cholesterol synthesis, propionate (13) increases gluconeogenesis and cholesterol synthesis in the liver, and butyrate (14) improves gut barrier function and mucin excretion, inhibits nuclear factor- κ B inflammatory cytokine transcription, and it's the preferred endothelial energy source.

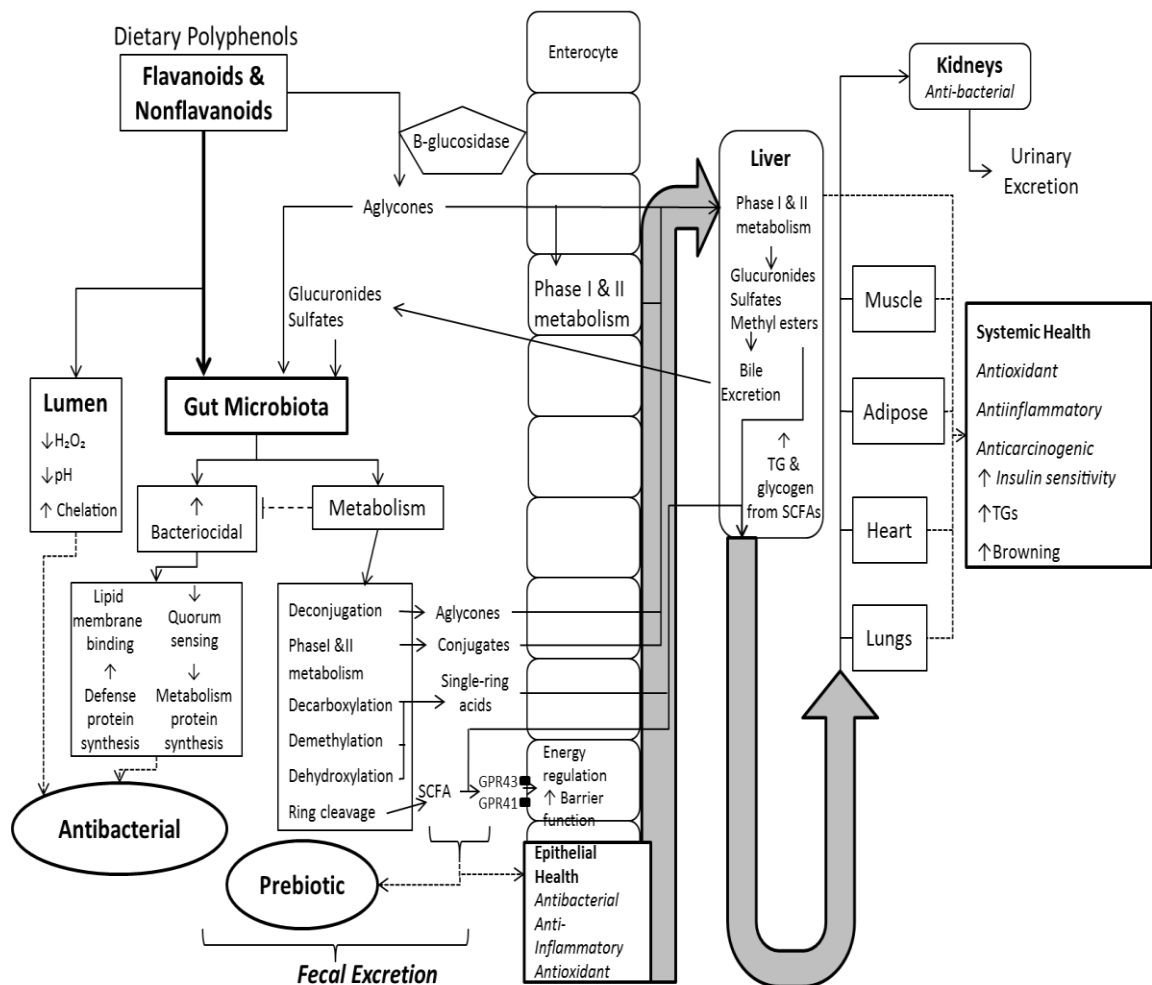


Figure 2.2. Polyphenol Metabolism and Health Benefits. Polyphenols are poorly absorbed and thus come in direct contact with gut microbes in the lower GI tract. Although some polyphenols are deconjugated in the small intestine into aglycones, which may passively diffuse into the enterocyte, the majority persist to the distal small intestine and colon. Within the lumen, polyphenols can indirectly influence microbial populations through reducing the pH and hydrogen peroxide (H_2O_2) levels, while also chelating with unabsorbed metal ions, thus negatively influencing pathogenic and some gram-positive bacterial growth. Polyphenols can be toxic to bacterial cells through disrupting normal cell properties, thus there are metabolized to

reduce the level of toxicity. Some metabolites may be used as energy sources (e.g., SCFAs) promoting beneficial microbial growth, whereas others diffuse through enterocytes or interact within the enterocyte (having beneficial effects). Absorbed polyphenol metabolites and aglycones enter the liver through the portal vein before entering the systemic circulation. Aglycones undergo further metabolism, once again becoming conjugated to make them easier to transport and excrete including transport back into the intestinal lumen via bile acid release from the gall bladder. As polyphenols and metabolites travel through systemic circulation, they may interact with various tissues or be excreted in the urine.

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CHAPTER III

CALIFORNIA TABLE GRAPE CONSUMPTION REDUCES ADIPOSITY, HEPATIC TRIGLYCERIDES, LIPOGENIC GENE EXPRESSION, AND ABUNDANCE OF SULFIDOGENIC BACTERIA IN MICE FED BUTTER FAT

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Abstract

The objective of this study was to examine the extent to which consuming polyphenol-rich, California table grapes reduces adiposity, hepatic steatosis, markers of inflammation or lipid metabolism, or impacts gut microbiota in mice fed a butter-rich diet. Male, C57BL/6J mice were fed a low fat diet or butter-rich diet with or without 3% or 5% grapes for 11 weeks. Total body and inguinal fat content were reduced in mice fed both levels of grapes compared to their high-fat, sugar controls. Mice fed 5% grapes had lower liver weights and triglyceride levels, and decreased hepatic expression of lipogenic glycerol-3-phosphate acyltransferase (*Gpat1*) compared to 5% controls. Mice fed 3% grapes had lower hepatic mRNA levels of the lipogenic genes peroxisome proliferator-activated receptor gamma 2, sterol-CoA desaturase 1, fatty-acid binding protein 4, and *Gpat1* compared to 3% controls. In white adipose tissue (WAT), mice fed 5% grapes had decreased mRNA levels of the lipogenic gene acylglycerol-3-phosphate-O-acyltransferase 2 compared to controls. Although grape feeding had only a minor impact on markers of inflammation in WAT or intestine, 3% grapes decreased the intestinal abundance of sulfidogenic *Desulfobacter* spp., and the *Bilophila wadsworthia*-specific dissimilatory sulfite reductase gene, and tended to increase the abundance of the beneficial bacterium *Akkermansia*

muciniphilia compared to controls. In addition, via 16s rRNA sequencing of cecum mucosa, Bifidobacterium, Lactobacillus, Allobaculum, and other genera were found to be negatively correlated with body fat percentage and inguinal fat weight. Allobaculum in particular was increased in both the LF and 3% grapes groups. Notably, grape feeding attenuated the high-fat induced impairment in localization of the intestinal tight junction protein zonula occludens. Collectively, these data indicate that some of the adverse health consequences of consuming a diet rich in saturated fat can be attenuated by table grape consumption.

Introduction

Currently 35.9% of adults and 17% of youth in the United States have been diagnosed as obese [1]. Obesity is also a global health condition affecting more than 10% of adults worldwide [2]. Of growing concern is the association between the rise in obesity and chronic inflammatory conditions such as type 2 diabetes, hypertension, and cardiovascular disease [3]. Physiologically, obesity is the result of an expansion of white adipose tissue (WAT) which typically elicits inflammatory signals involved in the recruitment of macrophages and other immune cells into the WAT. This results in a proliferation of circulating and tissue levels of proinflammatory cytokines and chemokines [4]. While increased tissue levels of these proinflammatory agents perpetuates the inflammatory cycle, those released systemically impair glucose disposal and lipid metabolism and hemostasis that contribute to the development of metabolic diseases. However, the exact mechanisms which initiate WAT inflammation resulting from diet-induced obesity remain unclear.

Gut microbes have received much attention due to their potential involvement in the development of obesity [5], chronic inflammation [6-8], and insulin resistance [9]. Diets high in fat, particularly those rich in saturated fatty acids found in milk fat [10], have been implicated in

the reduction of gut barrier-protecting bacteria as well as increasing the abundance of deleterious bacteria including sulfidogenic bacteria [reviewed in 11]. A correlation exists between the extent of these changes in microbiota and the degree of obesity and insulin resistance in test subjects [12]. Furthermore, the main byproduct of sulfidogenic bacteria like *Bilophila wadsworthia* and *Desulfovibrionaceae* spp. is hydrogen sulfide gas, which is genotoxic and cytotoxic and positively correlated with development of ulcerative colitis, gut inflammation, irritable bowel syndrome, and colon cancer [10, 13, 14].

In contrast, changes to the intestinal microbiome can have beneficial effects. Indeed, research models have demonstrated that elimination of gut microbes [5-9, 11, 12, 15] or inoculation with specific pre- (e.g., non-digestible sugars, fiber, or polyphenols) or probiotics (e.g., *Lactobacillus acidophilus*, *Bifidobacterium* spp., *Akkermansia muciniphila*, *Clostridium trybutyricum*) [reviewed in 11, 16-19] can attenuate obesity or metabolic dysfunction. Polyphenols found in fruits and vegetables [20-22] are of particular interest as they are poorly absorbed in the upper gastrointestinal tract, and thus persist in the distal small intestine, cecum, and colon [23] where they may influence microbiota taxa and their metabolites [24]. In addition, their anti-inflammatory, anti-oxidant, or anti-microbial actions have been reported to positively influence gut microbes and inflammation [reviewed in 25].

Grapes are rich in polyphenols including anthocyanins [reviewed in 25], and thus may have beneficial effects on intestinal or systemic inflammation. The anti-inflammatory and anti-oxidant properties of California table grapes have been demonstrated in rats supplemented with 9 human servings (3%, w/w) of powdered table grapes for 18 weeks, resulting in lowered blood pressure, improved cardiac function, and reduced systemic inflammation and oxidative damage [26]. These effects correlated with increased cardiac peroxisome proliferator-activated receptor (PPAR) α/γ activity and decreased nuclear factor kappa B (NF- κ B) activity, along with reductions in cytokine

levels [27]. Similarly, table grape-mediated reductions in atherosclerotic lesion areas were associated with increased serum antioxidant status and decreased macrophage-mediated oxidation of low density lipoproteins (LDL) [28]. Additionally, when women supplemented with powdered California table grapes (36 g/d) their plasma lipid profile improved and plasma (e.g., tumor necrosis factor alpha, TNF α) or urine (e.g., prostaglandin F2 alpha) markers of inflammation and oxidative stress were reduced [29]. Consistent with these data, we demonstrated that C57BL/6J mice fed a high fat diet (60% kcals from lard) supplemented with powdered California table grapes (3%, w/w), improved glucose tolerance at 5 weeks, and decreased markers of inflammation \approx 20-50% in serum and WAT at 18 weeks without affecting body fat levels or food intake [30].

Collectively, these data indicate that; (i) gut microbiota are influenced by diets abundant in saturated fats such as butter fat, in a manner that impacts gut inflammation and barrier function, and systemic inflammation, (ii) non-digestible carbohydrates and polyphenols positively impact adverse outcomes caused by high fat-feeding, and (iii) grape consumption can attenuate inflammation and oxidative stress in several animal models and in humans. However, little is known about how California table grapes decrease chronic inflammation associated with saturated fat-feeding and obesity. Therefore, the objective of this study was to examine the extent to which consuming California table grapes reduces adiposity, hepatic steatosis, markers of inflammation or lipid metabolism, or impacts gut microbiota in mice fed a butter-rich diet.

Materials and Methods

Animal

Four-week old, male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and acclimated on a standard chow diet for 1 week. Mice were housed in pairs, maintained at a temperature of 22°C with 50% humidity, and exposed to a 12 h light/ 12 h dark

cycle. Mice received food and water *ad libitum* and measures of body weight and food intake were conducted once and twice per week, respectively. All experimental procedures were performed under ethical standards and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Greensboro.

Diets

Animals were randomly assigned to one of five dietary treatments (n=10 per treatment group) as follows: low fat (LF; 10% of energy from fat), high fat (HF; 34% of energy from fat) plus 3% powdered grapes (w/w; HF-3G), high fat plus 3% sugar (w/w; HF-3S), high fat plus 5% powdered grapes (HF-5G) and high fat plus 5% sugar (HF-5S). The high fat diets consisted of approximately 3% energy from soybean oil and 31% energy from butter. Thus, the HF diets were rich in fat, especially saturated fat, and mimicked the average calories from fat in an American-type diet. The lyophilized (i.e., powdered) California table grapes, kindly provided by the California Table Grape Commission consisted of a mixture of red, green, and purple seeded and seedless grapes. The 3% and 5% dietary levels of grapes were comparable to 9 and 15 human servings (1 serving is equivalent to 1 cup of whole berries) of grapes, respectively. The HF-3S and HF-5S diets consisted of a mixture of fructose and glucose to control for the natural sugar content of the powdered grape diets. Detailed composition of the diets is illustrated in **Table 3.1**.

Intraperitoneal glucose tolerance tests (GTT) and fasting insulin levels

Intraperitoneal (i.p.) GTT were performed on weeks 3, 6, and 9 on non-anesthetized mice. Mice were fasted for 8 h and given an i.p. injection of glucose (i.e., 20% solution at 1 g/kg body weight). Blood from the tail vein collected at baseline and 5, 15, 30, 60, and 120 minutes post-i.p. glucose injection was used to quantify glucose levels using a Bayer Contour blood glucose monitor and strips (Bayer Healthcare, Tarrytown, NY, USA). Plasma insulin levels were detected

using an ultrasensitive mouse insulin kit (Crystal Chem, Inc, Downers Grove, IL). The homeostasis model assessment method (HOMA) for insulin resistance (IR) was used employing the following formula: [fasting insulin concentration (ng/ml) x 24 x fasting glucose concentration (mg/dl)] / 405 [16].

Body fat measurements via Dual X-Ray Absorptiometry (DEXA)

Percent body fat was measured using DEXA on a GE Lunar Prodigy Advanced System (GE Healthcare, Milwaukee, WI) at weeks 5 and 10. During the measurement, mice were lightly anesthetized with isoflurane using a SomnoSuite Small Animal Anesthesia System with Integrated Digital Vaporizer isoflurane system. Measurements were taken in duplicate to reduce the possibility of error and values expressed are an average of the two measurements.

Tissue collection

After 11 weeks of dietary intervention, mice were fasted for 8 h and euthanized via isoflurane-induced anesthesia followed by decapitation. Plasma was collected at time of harvest. Four white adipose tissue (WAT) depots were collected; epididymal, mesenteric, inguinal, and retroperitoneal. Additionally, livers were harvested and intestinal mucosa and digesta were collected from the duodenum, jejunum, ileum, cecum, and proximal and distal colon. Weights of the WAT depots and liver were recorded and all collected samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Liver and serum triglyceride (TG) levels

Liver TG content was measured as previously described [31]. Plasma TG content was determined using a commercial assay from Thermo Scientific and was conducted following the

manufacturer's protocol (Infinity TG assay #TR22421 and TG standards #TG22923; Norcross, GA).

RNA extraction and qPCR

Adipose and intestinal samples were homogenized in QIASol reagent and total RNA was extracted using QIAgen mini lipid kit obtained from Qiagen (Valencia, CA). For hepatic samples, a QIAgen mini universal kit from Qiagen was used. The quality and concentration of RNA were examined using absorbance at 260 nm and integrity determined using the absorbance ratio of 260/280 on a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA was created by reverse transcription using 1 ug of RNA and a high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocols. qPCR was performed in a 7500 FAST Real Time PCR system (Applied Biosystems). The expression of different genes related to inflammation, lipogenesis, and lipolysis in WAT depots and liver were measured using Taqman Gene expression assays purchased from Applied Biosystems. TATA-binding protein (*Tbp*) was the endogenous reference gene utilized for all assays and fold differences in gene expression were calculated as $2^{-\Delta\Delta Ct}$.

Immunoblotting

Immunoblotting was conducted as previously described [31] using primary antibodies for sterol-CoA desaturase-1 (#2283S; SCD1, Cell Signaling), carnitine palmitoyltransferase 1A (#12252S; CPT1a, Cell Signaling), peroxisome proliferator-activated receptor gamma (#2443S; PPAR γ Cell Signaling), β -actin (#4967; Cell Signaling), proliferator-activated receptor alpha (#sc9000; PPAR α , Santa Cruz Biotechnology Inc., Santa Cruz, CA), glycerol-3-phosphate acyltransferase (#sc382257; GPAM, Santa Cruz), and sterol regulatory element-binding protein 1C (#sc366; SREBP1c, Santa Cruz) all at dilutions of 1:1000. Adipocyte fatty acid binding

protein (aP2) was kindly provided by Dr. David Bernlohr (U. of Minnesota) and used at a 1:10,000 dilution. Horseradish peroxidase-conjugated secondary antibodies were probed for 2 h at room temperature at 1:1000 dilutions. Blots were exposed to a chemiluminescence reagent and X-ray films were developed using a SRX-101A Konica Minolta film developer.

Barrier function in ileum

The localization of the tight junction protein zonula occludens-1 (ZO-1) was determined in ileum samples that had been embedded in Tissue-Tek Cyro-OCT compound, sliced in 5 mm sections, fixed in cold methanol, and incubated with a polyclonal rabbit anti-ZO-1 antibody as previously described [32].

PCR amplification of 16S rRNA and functional gene targets

Real-time quantitative PCR (qPCR) was performed with a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). 16S rRNA gene-specific primers were used to target specific bacterial genera; *Akkermansia muciniphila*, *Bilophila wadsworthia* (F-AAGTCCTTCGGGCGAGTAA) (R-ATCCTCTCAGACCGGCTAC), *Desulfovibrio* spp. (DSV), *Desulfobulbus* spp. (DBB), *Desulfobacter* spp. (DSB), and *Desulfotomaculum* spp. (DFM) [4]. The *B. wadsworthia*-specific dissimilatory sulfite reductase (*dsrA-Bw*) [20], which encodes an enzyme that catalyzes a step in the reduction of inorganic sulfate to hydrogen sulfide, was targeted to measure the abundance of this member of the Desulfovibrionaceae family. Four functional genes utilized by *Fusobacterium nucleatum* in the fermentation of cysteine were measured; i.e., two homologues of L-cysteine desulfhydrase (FN0625 and FN1220), Cystathionine- β synthase (FN1055), and L-methionine- γ -lyase (FN1419). The abundance of butyrate-producing bacteria was also measured using the degenerate primer Butyrl-CoA transferase. Standard curves were constructed using cloned 16S rRNA and functional genes or

amplified PCR product. Genomic DNA extraction and PCR experiments were conducted as previously described [4].

Sequencing of 16S rRNA gene using Illumina Mi-Seq platform

To assess bacterial community structure, primers specific for 16S rRNA V4-V5 region (Forward: 338F: 5'-GTGCCAGCMGCCGCGGTAA-3' and Reverse: 806R: 5'-GGACTACHVGGGTWTCTAAT-3') that contained Illumina 3' adapter sequences as well as a 12-bp barcode were used. Sequences were generated by an Illumina MiSeq DNA platform at Argonne National Laboratory and analyzed by the program Quantitative Insights Into Microbial Ecology (QIIME) [33]. Operational Taxonomic Units (OTUs) were picked at 97% sequence identity using open reference OTU picking against the Greengenes database (05/13 release) [34]. OTUs were quality filtered based on default parameters set in the open-reference OTU command in QIIME and sequences were rarified to an equal sampling depth of 15000 reads per sample. Representative sequences were aligned via PyNAST [33], taxonomy assigned using the RDP Classifier [35], and a phylogenetic tree was built using FastTree [36]. Beta Diversity is represented by measuring UniFrac distances calculated using both weighted and unweighted algorithms and visualized with PCoA plots generated in Emperor. Prior to statistical analyses, OTUs occurring in less than 50% of samples were filtered from the OTU table. Significant changes in OTU abundance were assessed using Kruskal Wallis test (FDR correction $p \leq 0.05$). Multivariate statistical tests include ADONIS, ANOSIM, and PERMANOVA tests [33]. Spearman correlations and Principal Component Analysis (PCA) were run using MATLAB software.

Statistical Analysis

Data were analyzed using a one-way ANOVA and Student's *t* test to compute individual pairwise comparisons of means ($p < 0.05$). We also used Bonferroni's posthoc test to perform specific comparisons where appropriate. Analyses were conducted using the JMP software program version 10.0 for Windows (SAS, Cary, NC). Relative abundances generated from 16s rRNA sequencing were analyzed in GraphPad Prism Version 6 using ANOVA followed by Dunn's test for multiple comparisons. Data are expressed as means + S.E.M.

Results

Grape consumption decreases body fat

Body weight gains and energy intakes were greater in all HF-fed mice compared to the LF control mice (**Table 3.2**). Mice fed powdered grapes (HF-3G, HF-5G) had similar body weights and energy intakes compared to their HF-sugar controls (HF-3S, HF-5S; Table 3.2). Mice fed the HF-sugar control diets had greater body fat percentage and total WAT depot weights compared to the LF controls (**Fig. 3.1**). Mice fed the HF-3G diet had lower percent body fat at week 5 and mice fed powdered grapes at both levels had lower body fat percentages and inguinal fat depot weights at week 10 compared to their respective HF-sugar controls (Fig. 3.1). The HF-3G diet had lower total WAT depot weights compared to HF-3S controls (Fig. 3.1).

GTT, fasting glucose, insulin, and TG levels, and HOMA-IR scores unaffected by grapes

To assess the impact of grape consumption on insulin resistance and glucose sensitivity, GTT were conducted at weeks 3, 6, and 10, and fasting plasma glucose and insulin and HOMA-IR were measured at week 11. Mice consuming the HF-sugar control diets had impaired GTT at all three time points compared to the LF controls (**Fig. 3.2**). However, consuming grapes did not significantly improve GTT, fasting insulin, glucose, serum TG levels, or HOMA-IR scores.

Grape consumption lowers hepatic TG levels and the expression of several lipogenic genes

Liver tissue was analyzed to assess the impact of grape consumption on liver TG levels and markers of lipogenesis and fatty acid oxidation. Mice consuming the HF-sugar control diets had greater liver weights, TG levels (HF-5S only), and mRNA levels of the lipogenic genes *Ppar γ 2*, *Scd1* (HF-5S only), *Srebp1c*, cluster of differentiation 36 (*Cd36*; HF-5S only), and glycerol-3-phosphate acyltransferase 2 (*Gpat2*; HF-3S only) compared to the LF-fed mice (**Fig. 3.3**). Mice fed the HF-3G diet had decreased mRNA levels of *Ppar γ 2*, *Scd1*, fatty acid binding protein 4 (*Fabp4*), and *Gpat1* compared to the HF-3S controls. Mice fed the HF-5G diet had decreased TG levels and mRNA levels of *Gpat1* compared to the HF-5S controls. None of the genes associated with fatty acid oxidation (e.g., *Ppara*, *Cpt1a*, acyl-CoA oxidase 1 (*Acox1*)) were impacted by grape feeding. Consuming grapes had no impact on the protein levels of *Ppar γ* , *Scd1*, *Ppara*, *Fabp*, *Cpt1a*, or *Gpat* (data not shown).

Grape consumption differentially impacts WAT genes associated with inflammation and lipid metabolism

To determine whether insulin resistance caused by HF-feeding was due to WAT inflammation, inguinal (subcutaneous), epididymal (visceral), and mesenteric (visceral) WAT mRNA levels for several proinflammatory genes (i.e., cluster of differentiation 11c (*Cd11c*), epidermal growth factor-like module containing mucin-like hormone receptor 1 (*Erm1*; F4/80 human orthologue), monocyte chemoattractant protein 1 (*Mcp1*), *Tnfa*, Toll-like receptor 4 (*Tlr4*), and interleukin 6 (*Il6*) were measured. In epididymal WAT, mice fed the HF-sugar controls had increased mRNA levels of *Tnfa* (HF-5S), *Cd11c* (HF-5S), *Il6*, and *Mcp1* (HF-5S) compared to the LF controls (**Fig. 3.4A**). Grape feeding had no impact on these genes. In inguinal depot WAT, only mice fed the HF-5S diet had increased inflammatory gene expression (i.e., *Mcp1*), which was decreased in

mice fed the HF-5G diet (Fig. 3.4A). In mesenteric WAT, inflammatory gene expression was not increased by HF-feeding.

To determine if the reduction in adiposity by grape consumption was due to alterations in the expression of genes associated with fat synthesis or oxidation, epididymal and inguinal WAT were analyzed for mRNA markers of; (i) lipogenesis (i.e., *Ppar γ 2*, *Srebp1c*, *Scd1*, acylglycerol-3-phosphate-O-acyltransferase 2 (*Agpat2*), fatty acid synthase (*Fas*), acetyl-CoA carboxylase (*Acc*), perilipin 1 (*Plin1*), *Gpat1*), (ii) lipolysis (i.e., adipose TG lipase (*Atgl*); and (iii) beta-oxidation (i.e., *Acox1*, *Cpt1b*, *Ppara*). In epididymal WAT, the mRNA levels of the lipogenic genes *Ppar γ 2*, *Agpat2*, and *Scd1* (HF-5S) were higher in the HF-sugar controls compared to the LF-controls, and similar to the HF-grape groups (**Fig. 3.4B**). In inguinal WAT, the mRNA levels of the lipogenic genes *Srebp1c* (HF-3S), *Scd1*, *Agpat2* (HF-5S), *Fas* (HF-5S), and *Gpat2* (HF-5S) were higher in the HF-sugar controls compared to the LF-controls (**Fig. 3.4C**). Mice fed the HF-5G diets had lower mRNA levels of *Agpat2* compared to HF-5S control. The mRNA levels of the fatty-acid oxidizing genes *Ppara* and *Cpt1b*, and the lipolytic gene *Atgl* (HF-3S) were lower in the HF-sugar controls compared to the LF control, and similar to the HF-grape groups.

Minimal influence of HF-feeding and grape consumption on markers of intestinal inflammation and barrier function

Given the reported adverse effects of consuming saturated fats [reviewed in 11], particularly from milk fat [10], on intestinal health, and potential prebiotic impact of grapes, we measured the effects of our diets on markers of intestinal inflammation and barrier function. For inflammatory status, the mRNA levels of *Cd11c*, *Erm1*, *Mcp1*, *Tnfa*, *Tlr4*, and *Il6* in ileum and distal colon mucosa and the activity of duodenal alkaline phosphatase and ileal myeloperoxidase were measured. Surprisingly, the only proinflammatory gene increased in the ileal mucosa of HF-sugar

fed mice was *Tnfa*, which was similar to the HF-5G group (**Fig. 3.5A**). None of the proinflammatory genes measured in the colonic mucosa were increased by HF-sugar feeding or grape consumption. Similarly, the activities of alkaline phosphatase and myeloperoxidase were not influenced by HF-sugar feeding or grape consumption (data not shown).

To assess intestinal barrier function, the mRNA levels of the tight junction proteins zonula occludens (*Zo1*), claudin-1, and occludin-1 and the localization of ZO-1 at the apical surface of the ileal epithelium were measured. Although the ileum mucosal gene expression of these tight junction proteins was not impacted by the diets, the localization of ZO-1 was impaired in the HF-sugar control diets compared to the LF controls, and improved by grape feeding (**Fig. 3.5B**).

16s rRNA sequencing of the gut microbiota reveals genera associated with body fat percentage and inguinal fat pad weight

Given the reported adverse effects of consuming saturated fats [reviewed in 11], particularly from milk fat [10], on intestinal microbes, we measured the effects of our diets on the abundance of several mucosal sulfidogenic bacteria (i.e., *DBB*, *DSB*, *dsrA*, *DFM*, *DSV*, and *B. wadsworthia*) or their gene products (i.e., *B. wadsworthia* specific *dsrA-Bw*, *Fusobacterium nucleatum* functional genes including two L-cysteine desulfhydrases (*FN0625* and *FN1220*) cystathionine- β synthase (*FN1055*), and L-methionine- γ -lyase (*FN1419*)). Although HF-feeding did not increase the expression of any of these genes associated with sulfur metabolism in ileum or colon mucosa compared to LF control mice (data not shown), mice consuming the HF-3G diet had decreased mRNA levels of *DSB* and *dsrA-Bw* expression in ileum mucosa compared to mice consuming the HF-3S diet (**Fig. 3.6A**).

We also measured the expression of a functional gene (i.e., butyryl-CoA transferase) of health-promoting, butyrate-producing bacteria and on the abundance of *Akkermansia muciniphila*, a

mucin-degrading bacteria associated with prebiotic-mediated reduction in obesity [37]. Although not statistically significant, mice consuming grapes had increased levels of *Akkermansia muciniphila* in the cecum digesta (HF-3G), colon digesta (HF-3G), and proximal colon mucosa (HF-5G) compared to their HF-sugar controls (**Fig. 3.6B**).

Sequencing of the 16s rRNA gene reveals alterations in microbial structure and relationships

In order to better understand the impact of grape feeding on gut bacterial community structure, an untargeted approach was used by sequencing the 16s rRNA gene in cecum mucosal samples. Sequencing was performed on an Illumina MiSeq platform using primers targeting the V4-V5 region of the 16s rRNA gene. Data were analyzed using QIIME 1.8 Software (33-Caporaso et al. 2010). Alpha Diversity analyses revealed that the HF groups containing 5% grapes or 5% sugar resulted in reduced observed species compared to 3% grapes ($p = 0.0002$) or 3% sugar ($p < 0.0001$), respectively (**Fig 3.7**). Notably, HF diet containing 3% sugar reduced observed species compared to 3% grapes ($p=0.0026$), but there was no difference in observed species in 5% grape and sugar groups. These results suggest that elevated sugar content may result in decreased alpha diversity or membership of the gut microbiota. Principal Coordinate Analysis (PCoA) of weighted UniFrac distances did not reveal obvious differences in beta diversity or community structure across diet groups but there was clear separation between the LF group and HF groups based on unweighted UniFrac distances (**Fig. 3.8**). To further interrogate differences between diet groups, multivariate statistical tests including ADONIS, ANOSIM, and PERMANOVA were conducted. Significant differences were found among both unweighted and weighted UniFrac distances across diet groups (**Table 3.3**). Subsequently, OTUs that were not represented in up to 50% of the samples were removed to reduce noise from low abundance OTUs. Next, a Kruskal Wallis test was performed to determine significant differences in relative abundance of taxa

between groups. Here, it was found that several bacterial taxa were significantly altered based on diet (**Fig. 3.9**). While many genera were reduced in the HF diets compared to the LF diet, some were selectively increased in HF-3G group such as *Ruminococcus* and *Anaeroplasma* in the Firmicutes and Tenericutes phylum, respectively (Fig. 3.9).

To assess differences between specific diet groups, Principal Component Analysis (PCA) was conducted and clear clustering was evident between the LF and HF-3G group (**Fig. 3.10A**) and between the HF-3G and HF-3S group (**Fig 3.10B**). Principal Component Analysis between the LF and HF-3G group revealed that ~60% of the variance in relative bacterial abundance between the two groups is explained with Principal Component 1 (PC1). PC1 exhibits strong correlations with two strains of bacteria: S24-7 within the Bacteroides phylum and *Clostridiales* in the Firmicutes phylum (Fig. 3.10A). This indicates that the variance explained by PC1 is primarily due to the relative abundances of these two bacterial strains. Principal component 2 (PC2) explained an additional 20% of the variance between groups and was primarily correlated with *Akkermansia*. PCA between the HF-3G and HF-3S groups revealed that ~60% of the variance in relative microbial abundance between the two groups was explained by PC1. PC1 was once again strongly correlated with S24-7 and *Clostridiales* (Fig. 3.10B). PC2 explained ~20% of the variance and exhibited a strong correlation with *Akkermansia*, however this was positively associated with the HF-3S group. These data suggest that most variance between the LF and HF-3G was explained by the abundance of S24-7 in the LF group. Interestingly, S24-7 also explained the variance between the HF-3G and HF-3S groups, as it was positively correlated with HF-3G compared to HF-3S. These data may suggest that S24-7 is associated with improved metabolic profile of the LF and HF-3G groups.

Lastly, Spearman Correlation analysis revealed significant negative correlations between body fat percentage and several genera (**Fig. 3.11, Table 3.4**) with the most profound being

Bifidobacterium ($p = 0.0001$), a butyrate producer, and others including *Lactobacillus* ($p = 0.0221$) and *Allobaculum* ($p = 0.034$; Fig 3.11, Table 3.4). These particular genera were similarly correlated with inguinal fat pad weight. *Clostridiales* of the Firmicutes phylum was positively correlated with body fat percentage ($p = 0.0065$). Interestingly, a heatmap made from the taxa that were significantly correlated with body fat percentage, reveals that *Allobaculum* belonging to the Tenericutes phylum was increased in both the LF and HF3G group (**Fig. 3.11**). Little is known regarding the functional role of *Allobaculum* in the gut ecosystem and is currently being investigated for its potential association with a lean phenotype and improved metabolic health.

Discussion

Our data demonstrate that consuming table grapes (i.e., 3-5%, w/w; equivalent to 9-15 human servings) attenuates the accumulation of body and liver fat in mice fed a diet rich in butter compared to control mice. However, these lipid-lowering effects of grapes were not associated with improvements in glucose tolerance or markers of inflammation in intestinal mucosa or WAT. Notably, the impaired localization of the intestinal tight junction protein ZO-1 in high fat-fed mice was improved by grape consumption. Populations of the deleterious sulfidogenic bacteria *Desulfobacter* spp, and the *Bilophila wadsworthia*-specific dissimilatory sulfite reductase gene were decreased by grape consumption (HF-3G), and populations of the beneficial bacterium *Akkermansia muciniphila* tended to be higher the colonic mucosa (HF-5G) or digesta (HF-3G) of grape fed mice compared to their respective controls. Additionally, via 16s rRNA sequencing analysis, we found significant differences across groups in the relative abundance of S24-7 and *Akkermansia* which were found to be negatively correlated with HF diet compared to the LF diet. Intriguingly, correlation analysis showed a strong negative correlation between *Bifidobacterium* and body fat percentage and inguinal fat pad weight. Taken together, these data indicate that

consumption of table grapes attenuates adiposity and steatosis that are positively correlated with a marker of intestinal integrity and changes in several species of gut microbes in mice fed a butter-rich diet.

Influence of dietary polyphenols on adiposity, steatosis, and glucose tolerance

Many studies have demonstrated that consuming diets rich in calories from fat, particularly saturated fat, and sugars promote obesity and its metabolic complications [reviewed in 11]. The ability for foods rich in polyphenols, including grapes, to prevent obesity-mediated inflammation or related disorders has been demonstrated [reviewed in 25]. For example, grape seed procyanidin extract supplementation reduces body weight gain and adipose tissue mass in hamsters fed a HF diet [38]. Additionally, mice fed a HF diet supplemented with muscadine grape phytochemicals rich in anthocyanins had decreased body weights, less lipid accumulation in the liver, and improved glucose tolerance compared to high fat controls [39].

While several studies demonstrate anti-inflammatory and anti-obesity effects of grapes or their extracts, the mechanisms by which these effects occur are less clear. Anthocyanins are one of the most abundant phytochemicals in grapes [31] and therefore may be responsible for mediating the reductions in adiposity and steatosis. For example, anthocyanins purified from purple sweet potatoes attenuated hepatic lipid accumulation via the activation of adenosine monophosphate-activated protein kinase and decreased expression of SREBP1 and its downstream target genes in mice fed a HF diet [40]. Our study found that powdered grape supplementation at 3% and 5% reduced body weight and fat gain and hepatic lipid accumulation, but had no significant effects on the expression of *Srebp1c* and *Fas*. One explanation for the differential effects of extracted anthocyanins cited above [40] and our anthocyanin-rich grapes may be due to the binding of anthocyanins to fibers or proteins in the intact grapes, making them less bioavailable to the host.

Although we discovered that the expression of several hepatic genes associated with lipogenesis (e.g., *Ppar γ* , *Scd1*, *Fabp4*, *Gpat1*) in liver were lower in mice consuming the HF-3G compared to the HF-3S controls, the protein levels of associated with these genes were not lowered by grape feeding. However, we did not measure the activity of these proteins, and therefore do not know they were impacted by grape consumption.

Relationship between dietary fat, sulfite-reducing bacteria, barrier function, and inflammation

Devkota et al. [10] demonstrated that feeding a milk-fat-based diet similar in amount and composition to the western diet (i.e., 37% kcals from fat) to IL-10 knockout mice decreased gut barrier-protecting bacteria and increased sulfate-reducing bacteria and increased intestinal markers of inflammation consistent with inflammatory bowel disease [10]. Mice consuming the milk fat diet had increased abundance of *B. wadsworthia*, which was associated with a pro-inflammatory T helper type immune response and increased colitis. Notably, taurine-conjugated bile salts caused these inflammatory responses, in part, due to their high sulfur content, which stimulates sulfite-reducing bacteria like *B. wadsworthia*. In a parallel 3 week study, these authors fed C57BL/6J mice the same high fat diet [10]. Consistent with the IL-10 knockout mice, C57BL/6J mice fed the milk fat diet had an increased bloom of the sulfidogenic bacteria *B. wadsworthia* and the *B. wadsworthia*-specific dissimilatory sulfite reductase gene *dsrA-Bw*. Milk fat-fed C57/6J mice also had a higher abundance of Bacteroidetes and a lower abundance of Firmicutes compared to the low fat-fed mice [10]. However, milk fat-fed C57BL/6J mice did not present overt colitis as did the IL-10 knockout mice [10]. In the present study, we did not observe any increases in the expression of sulfidogenic bacteria-related genes in the high fat-fed controls compared to the LF fed mice, although the HF-3G had lower expression levels of *dsrA-Bw* and *DSB* in the ileum mucosa compared to the HF-3S control group (Fig. 3.6A). This decreased

abundance of sulfidogenic bacteria correlated with improved localization of the ileal tight junction protein ZO-1 (Fig. 3.5). Although no markers of inflammation in the intestinal samples were significantly increased by the milk fat based diet or decreased by grape feeding, the expression levels of several inflammatory genes in visceral epididymal WAT (i.e., *Tnfa*, *Cd11c*, *Mcp1*) and subcutaneous inguinal WAT (i.e., *Mcp1*) were increased by HF feeding (Fig. 3.4A), and HF-5G consumption reduced *Mcp1* expression in inguinal WAT compared to its control HF-5S (Fig. 3.4A).

These results are comparable to data showing that HF feeding selectively increases the abundance of a specific type of bacteria that are associated with intestinal inflammation [41]. ApoA-I knockout mice, which present impaired glucose tolerance and increased adiposity, and wild type mice were fed a very HF diet (i.e., ~60% kcals from lard) for 25 weeks. HF fed mice had increased abundance of *Desulfovibrionaceae*, a family of sulfate/sulfite reducing bacteria that produce hydrogen sulfide, a genotoxic gas that causes barrier dysfunction and endotoxemia [42]. Consistent with these data, we showed that C57BL/6J mice fed a very HF diet (i.e., ~60% kcals from lard) for 20 weeks increased three types of sulfidogenic bacteria in colonic mucosa, impaired localization of ZO-1, and mRNA levels of markers of macrophage infiltration in intestinal mucosa and WAT compared to LF fed mice [32]. Collectively, these data demonstrate that consuming diets enriched with lard or butter, two fat sources containing high levels of saturated fatty acids, increases the abundance or markers of sulfate-reducing bacteria associated with impairment of intestinal barrier function and contribute to systemic inflammation.

Relationship between dietary fat, Akkermansia muciniphila, barrier function, and inflammation

Akkermansia muciniphila, a commensal, mucin-degrading bacteria, plays a role in preventing the development of diet-induced obesity [37]. Under normal conditions, these bacteria represent

3-5% of the gut microbial population in humans. However, HF feeding reduces the colonic abundance of these bacteria in mice 100-fold, and oligofructose prebiotic treatment prevents this loss. Oligofructose-mediated increase in *A. muciniphila* was linked to decreased metabolic endotoxemia and markers of inflammation in WAT. Furthermore, supplementation of C57BL/6J mice fed a HF diet with *A. muciniphila* for 4 weeks lead to a decrease in adiposity, gut barrier dysfunction, metabolic endotoxemia, glucose intolerance, insulin resistance, and *Cd11c* expression in WAT, and improved mucus thickness along the epithelium compared to control mice [37]. Consistent with these data demonstrating that oligofructose prebiotic treatment attenuates a HF diet-induced reduction of *A. muciniphila*, our data indicate that supplementing high fat, butter-rich diets with 5% California table grapes increases the abundance of *A. muciniphila* in proximal colon mucosa compared to the HF-sugar controls (Fig. 3.6B). This increase in *A. muciniphila* was positively associated with improved localization of ZO-1 in the apical area of the ileal epithelium compared to HF control mice (Fig. 3.5). Furthermore, based on our 16s rRNA sequencing data of the cecum mucosa, the genus *Akkermansia* was positively associated with mice fed a LF diet, further supporting a relationship with a lean phenotype.

Limitations and unanswered questions

The current study did not achieve the anticipated increases in intestinal and systemic inflammation in young C57BL/6J mice fed a high fat diet rich in milk-fat as demonstrated in IL-10 knockout mice fed milk-fat [10] or in ApoA-I knockout mice fed lard [40]. Perhaps feeding mice extremely HF diets (e.g., 60% kcals from lard) is necessary to instigate intestinal and systemic inflammation [11, 30]. Our current study sought to provide a more physiological approach to achieve diet-induced obesity accompanied by systemic and intestinal inflammation. The use of this more physiological model for the time span of 11 weeks may not have been aggressive

enough in this model to elicit a robust inflammatory response, as the percentage of calories from fat (34% of kcals) or the composition of fat (primarily milk fat) may not have been adequate, to induce a severe case of obesity and inflammation. Future studies should examine the effect of different polyphenols or fractions present in grapes on obesity and systemic and intestinal inflammation using a diet induced obesity mouse model fed a diet that is more representative of the fat composition of the typical American diet (i.e., a mixture of animal and vegetable fats consisting of beef tallow, lard, milk fat, shortening, and vegetable oils [43].)

Acknowledgements

We would like to acknowledge the contributions of everyone involved in collecting data for this study. Brian Collins and Jessie Baldwin equally conducted all stages of the animal study, including caring, feeding, and weighing of the mice, the glucose tolerance testing, and the measurement of body fat using DEXA. Brian Collins additionally measured markers of ileal and colonic gene and protein expression associated with inflammation, ileal activity of myeloperoxidase, and duodenal activity alkaline phosphatase. Jessie Baldwin additionally measured markers of inguinal and epididymal gene and protein expression associated with inflammation, fat depot and liver gene expression of markers of lipolysis and lipogenesis, and serum triglyceride levels. Robin Hopkins measured plasma insulin using an ELISA assay. Chia-Chi Chuang measured liver triglyceride levels. H.R. Gaskins and Patricia Wolfe together measured the PCR of 16s rRNA for sulfidogenic bacteria and *A. muciniphilia* markers. Kristina Martinez, Eugene Chang measured the sequencing of 16s rRNA using the Illumina Mi-seq platform and Chase Cockrell conducted the analyses of data shown in Figures 3.7-3.10. Wei Zhong measured the ileal localization of the tight junction protein ZO-1. Paula Cooney measured the body fat mass using DEXA (with the assistance of Brian Collins and Jessie Baldwin).

Table 3.1. Diet Formulations

Ingredients (gram)	LF	HF-3S	HF-3G	HF-5S	HF-5G
Casein	200	200	200	200	200
L-Cystine	3	3	3	3	3
Corn Starch	506.2	194	194	194	194
Maltodextrin 10	125	94	94	94	94
Fructose	0	13.3	0	21.9	21.9
Dextrose, Monohydrate	0	13.3	0	21.9	21.9
Sucrose	68.8	154	154	139	139
Cellulose	50	50	50	50	50
Soybean Oil	25	12	12	12	12
Butter	0	144	144	144	144
Lard	20	0	0	0	0
Mineral Mix	10	10	10	10	10
DiCalcium Phosphate	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5
Potassium Citrate	16.5	16.5	16.5	16.5	16.5
Vitamin Mix	10	10	10	10	10
Choline Bitartrate	2	2	2	2	2
Grape Powder	0	0	28.5	0	47
Total	1055.1	854.7	857.5	855.7	857.7

LF, low fat control; HF-3S, high fat- 3% sugar control; HF-3G, high fat- 3% grapes; HF-5S, high fat-5% sugar control; HF-5G, high fat-5% grapes

Table 3.2. The Effect of Diet Composition on Weight Gain, Consumption, and Utilization

Diets	BWG n=9-10	FI n=5	FCE n=5	Kcal n=5
LF	7.0 ± 0.6 ^b	399 ± 8 ^b	28.6 ± 1.2 ^a	1534 ± 26 ^b
HF-3G	13.1 ± 0.7 ^a	465 ± 7 ^a	17.8 ± 0.8 ^b	2051 ± 26 ^a
HF-3S	11.5 ± 0.7 ^a	424 ± 20 ^{ab}	18.7 ± 0.9 ^b	1874 ± 80 ^a
HF-5G	11.3 ± 1.1 ^a	409 ± 12 ^{ab}	18.4 ± 1.1 ^b	1799 ± 45 ^{ab}
HF-5S	12.6 ± 1.0 ^a	438 ± 25 ^{ab}	17.8 ± 1.5 ^b	1957 ± 124 ^a

Mean ± SEM without a common lower case letter in a column differ ($p < 0.050$ using one-way ANOVA and Student's t test. BWG = total body weight gain (g). FI= total food intake per cage (g). FCE= food conversion efficiency per cage. Kcal= total caloric intake per cage (kcal).

Table 3.3. Statistical Analyses of Unifrac Distances

Weighted	R2	P Value
Adonis	0.12361	0.001
Anosim	0.3048	0.01
	Pseudo F Statistic	p value
Permanova	1.4105	0.001
Unweighted	R2	P Value
Adonis	0.23825	0.001
Anosim	0.2609	0.01
	Pseudo F Statistic	p value
Permanova	3.1277	0.001

Table 3.4. Taxa Associated with Body Fat

Taxa Associated with Body Fat Percentage: (Phylum; Class; Order; Family; Genus; Species)	Correlation	P Value
Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium	-0.53	0.0001
Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	-0.33	0.0221
Firmicutes; Clostridia; Clostridiales; Other; Other	0.39	0.0063
Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Other	-0.32	0.0243
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus	-0.35	0.0147
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum	-0.30	0.0369
Taxa Associated with Inguinal Fat Pad Weight: (Phylum; Class; Order; Family; Genus; Species)	Correlation	P Value
Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium	-0.48	0.0004
Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	-0.39	0.0053
Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae; Leuconostoc	-0.29	0.0413
Firmicutes; Clostridia; Clostridiales; Other; Other	0.38	0.0065
Firmicutes; Clostridia; Clostridiales; Clostridiaceae	-0.34	0.0177
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Ruminococcus	-0.35	0.0131
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum	-0.30	0.0391
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Coprobacillus	0.31	0.0297
Tenericutes; Mollicutes; Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma	-0.30	0.0380

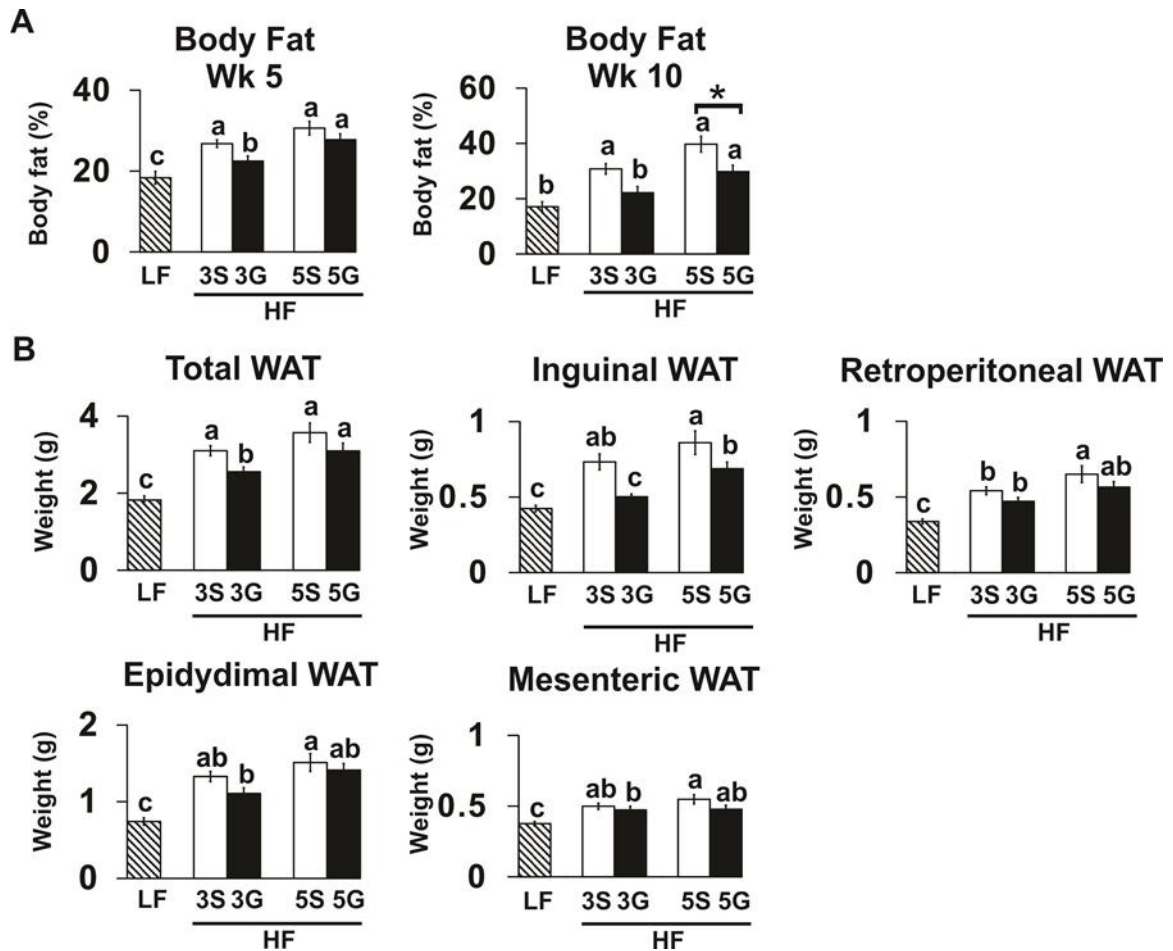


Figure 3.1. Diet Induced Changes to Fat Mass. Adiposity indices of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks.

(A) Body fat percentages were measured at week 5 and week 10 using dual energy x-ray absorptiometry (DEXA). (B) At week 11, epididymal, inguinal, retroperitoneal, and mesenteric white adipose tissue (WAT) depots were excised and weighed. The weights of the epididymal, inguinal, retroperitoneal, and mesenteric depots were measured, and their sum labelled total WAT. Means \pm SEM without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's t test. Means \pm SEM ($n = 9-10$) sharing the symbol "*" differ using the Bonferroni's adjustment ($p < 0.01$).; LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet

containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.



Figure 3.2. Glucose Tolerance Tests. Glucose tolerance tests (GTT) s of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. At weeks 3, 6, and 9, GTTs were conducted on mice fasted for 8 h and injected i.p. with a 20% glucose solution. Data are expressed as total area under the curve (AUC) for the GTTs. Means \pm SEM (n=9-10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

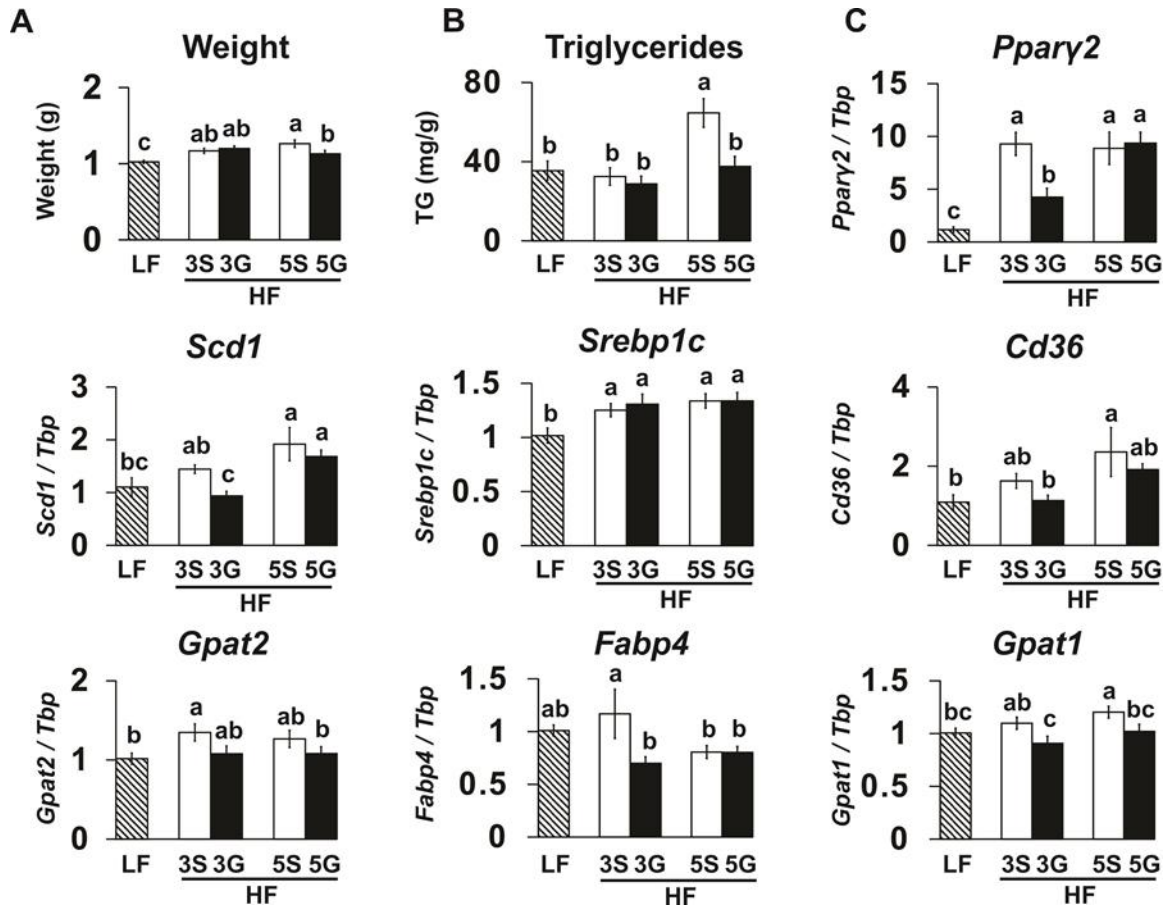


Figure 3.3. Liver Gene Expression. Liver weights, liver triglyceride levels, and the expression of several lipogenic genes in liver of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. qPCR was conducted to measure mRNA abundance of genes associated with hepatic lipogenesis. Means \pm SEM (n=9-10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

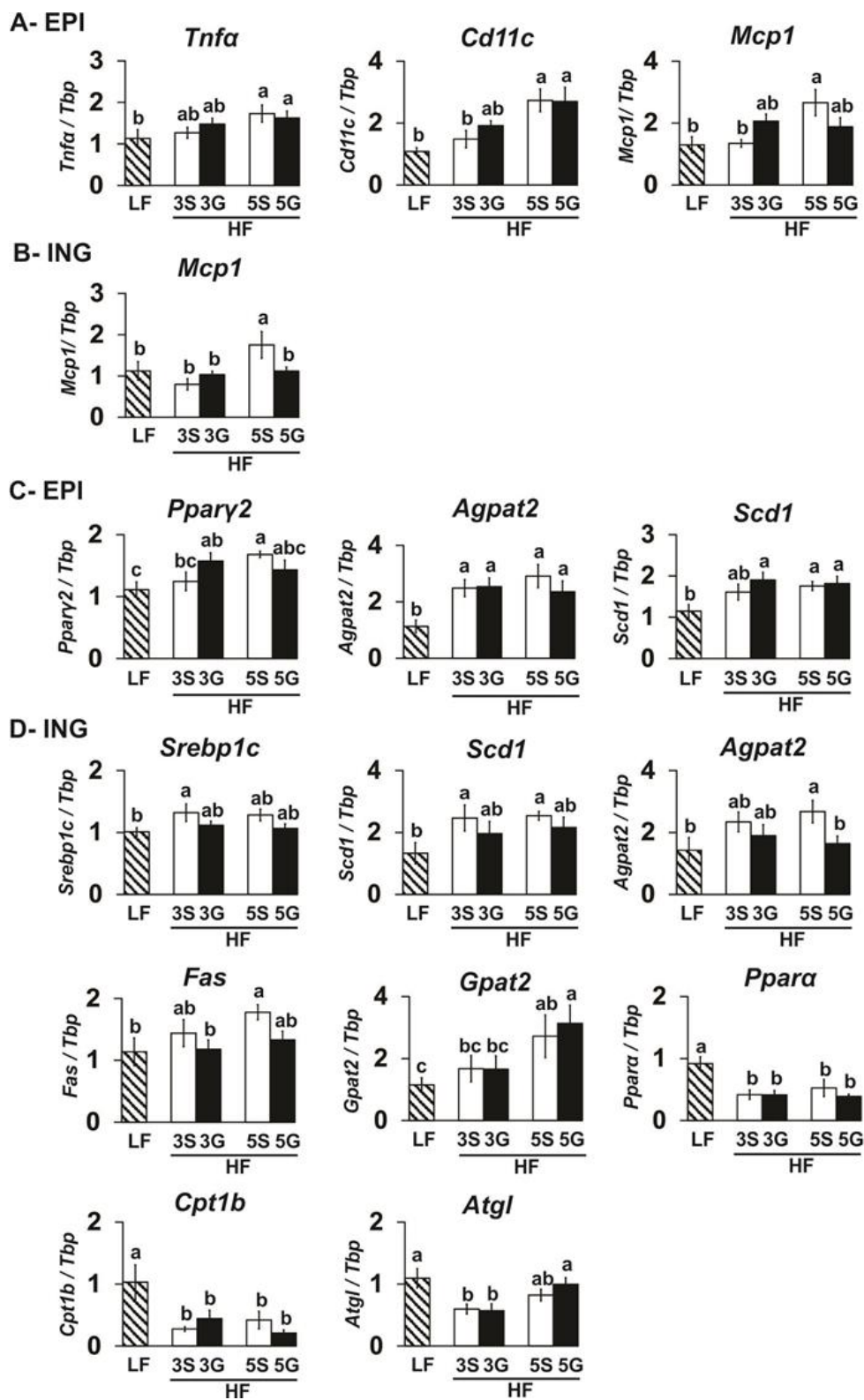
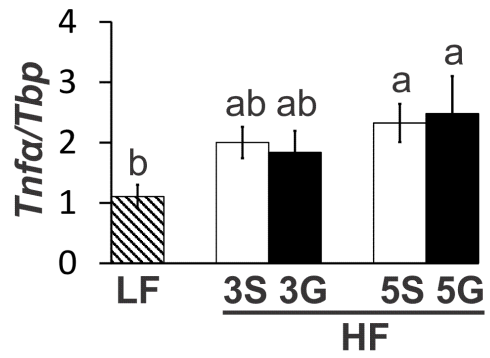


Figure 3.4. WAT Gene Expression. The expression of markers of inflammation and lipid metabolism in epididymal and inguinal WAT of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. qPCR was conducted to measure mRNA abundance of genes associated with (A) inflammation and lipid metabolism in (B) epididymal (EPI; visceral) and (C) subcutaneous (ING; subcutaneous) WAT depots. Means \pm SEM (n=9-10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

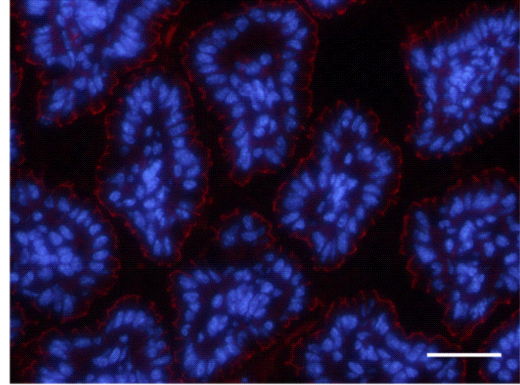
A-Ileum

Tnfa

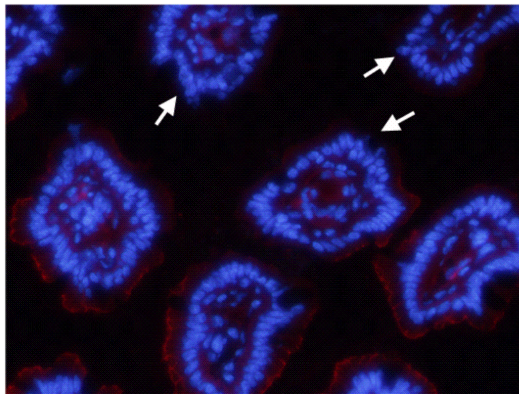


B-Ileum

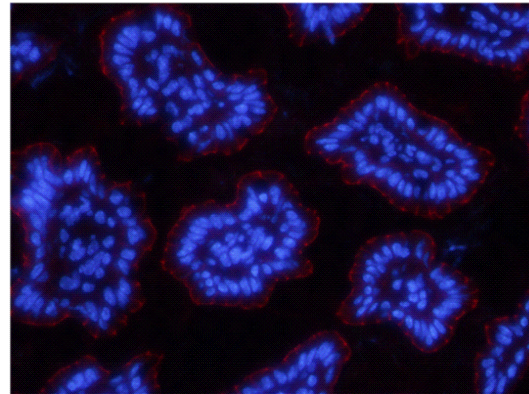
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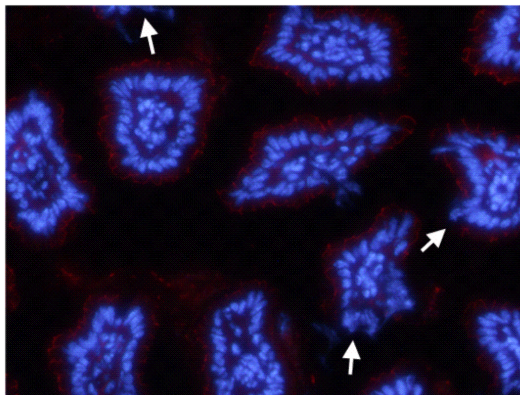
3S



3G



5S



5G

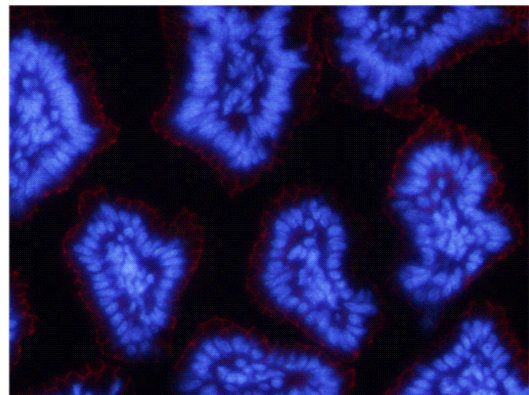
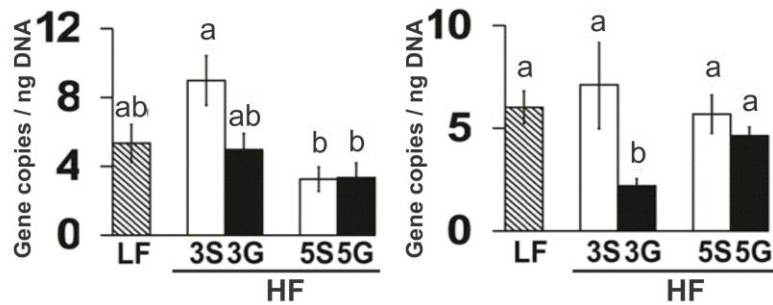
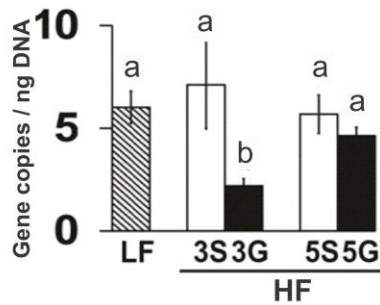


Figure 3.5. Alterations to Intestinal Inflammation and Barrier Function. (A) Ileum mucosa expression of TNF α and (B) the localization of the tight junction protein ZO-1 at the apical area of the ileum epithelium in C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. qPCR was conducted to measure mRNA abundance of inflammatory genes. Localization of ZO-1 was visualized by immunostaining of ileum samples (n=5). Means \pm SEM (n=9-10 for TNF α) without a common lowercase letter differ (p<0.05) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

A-Sulfidogenic Bacteria *dsrA Bw*

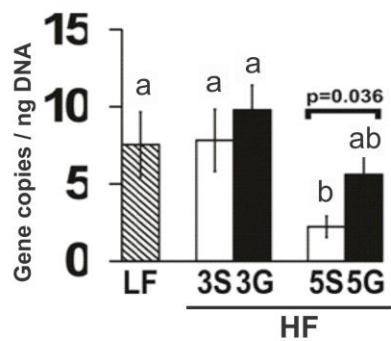


DSB



B-*Akkermansia muciniphila*

P. colon mucosa



Colon Digesta

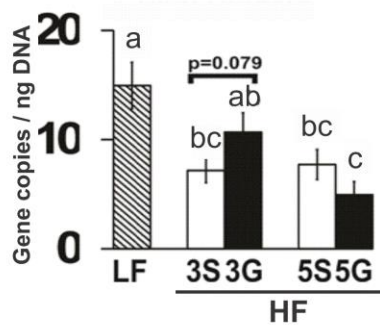


Figure 3.6. Diet Induced Alterations to Sulfidogenic Bacteria and *Akkermansia*

muciniphilia. Abundance of sulfidogenic bacteria and *Akkermansia muciniphilia* in the intestinal mucosa or digesta of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Abundance of the sulfidogenic, *Bilophila wadsworthia*-specific, functional gene target dissimilatory sulfate reductase (*dsrA-Bw*) and the targeted sulfidogenic bacterial genera *Desulfobacter* (DSB) species in the ileum mucosa. (B) The abundance of the probiotic *Akkermansia muciniphilia* in colon mucosa and digesta and in cecum digesta. qPCR was conducted to measure mRNA abundance of the functional genes and bacterial genera. Means \pm SEM (n=9-10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's t test. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat

diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes. Means \pm SEM (n=9-10) without a common lowercase letter differ ($p < 0.05$).

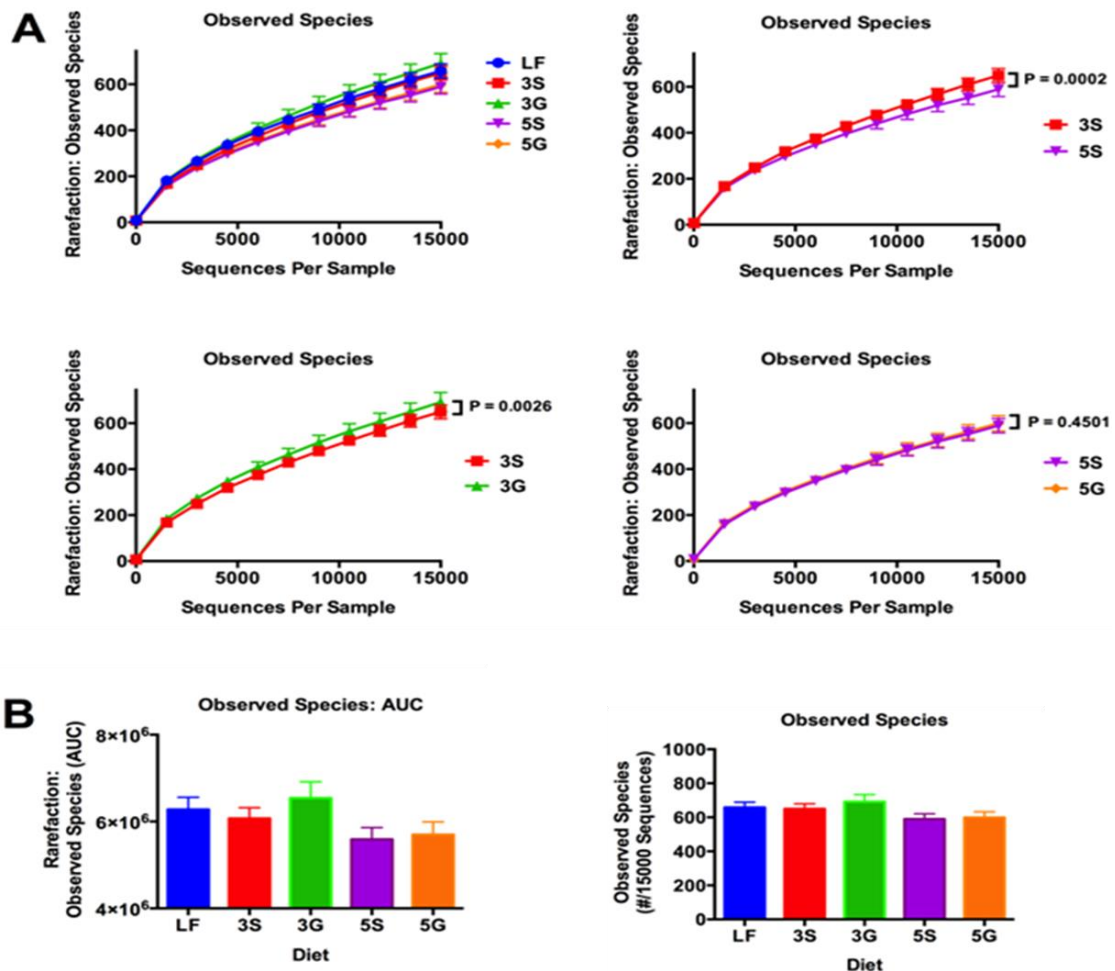


Figure 3.7. Diet Induced Changes to Microbial Species. Observed bacterial species in cecum mucosa of C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Rarefaction curves of observed species are shown. Samples were rarified to 15,000 sequencing reads per sample. (B) Above - Area Under the Curve (AUC) of rarefaction curves shown for each diet group. Below – Observed species at 15,000 sequencing reads for each diet group. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing

5% grapes. Data are presented as Means \pm SEM (n=9-10) using one-way ANOVA and Dunn's test for multiple comparisons.

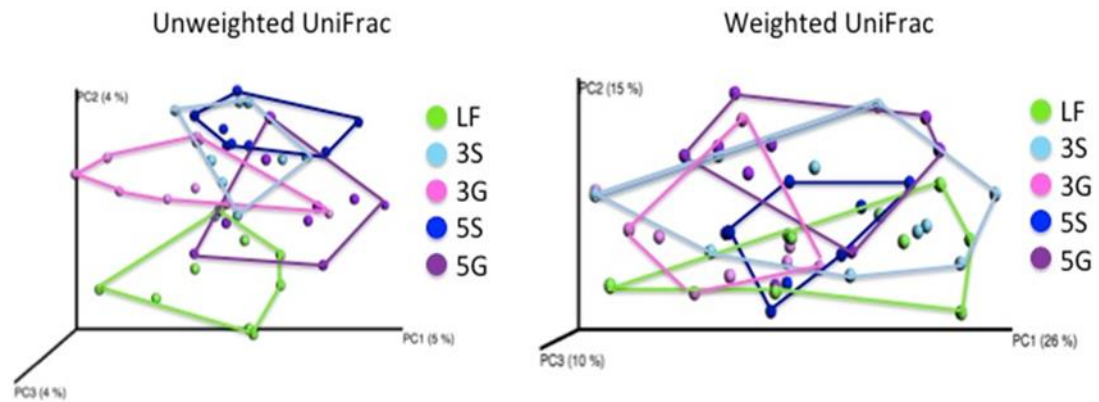


Figure 3.8. PCoA Plots of Unweighted and Weighted UniFrac Distances. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

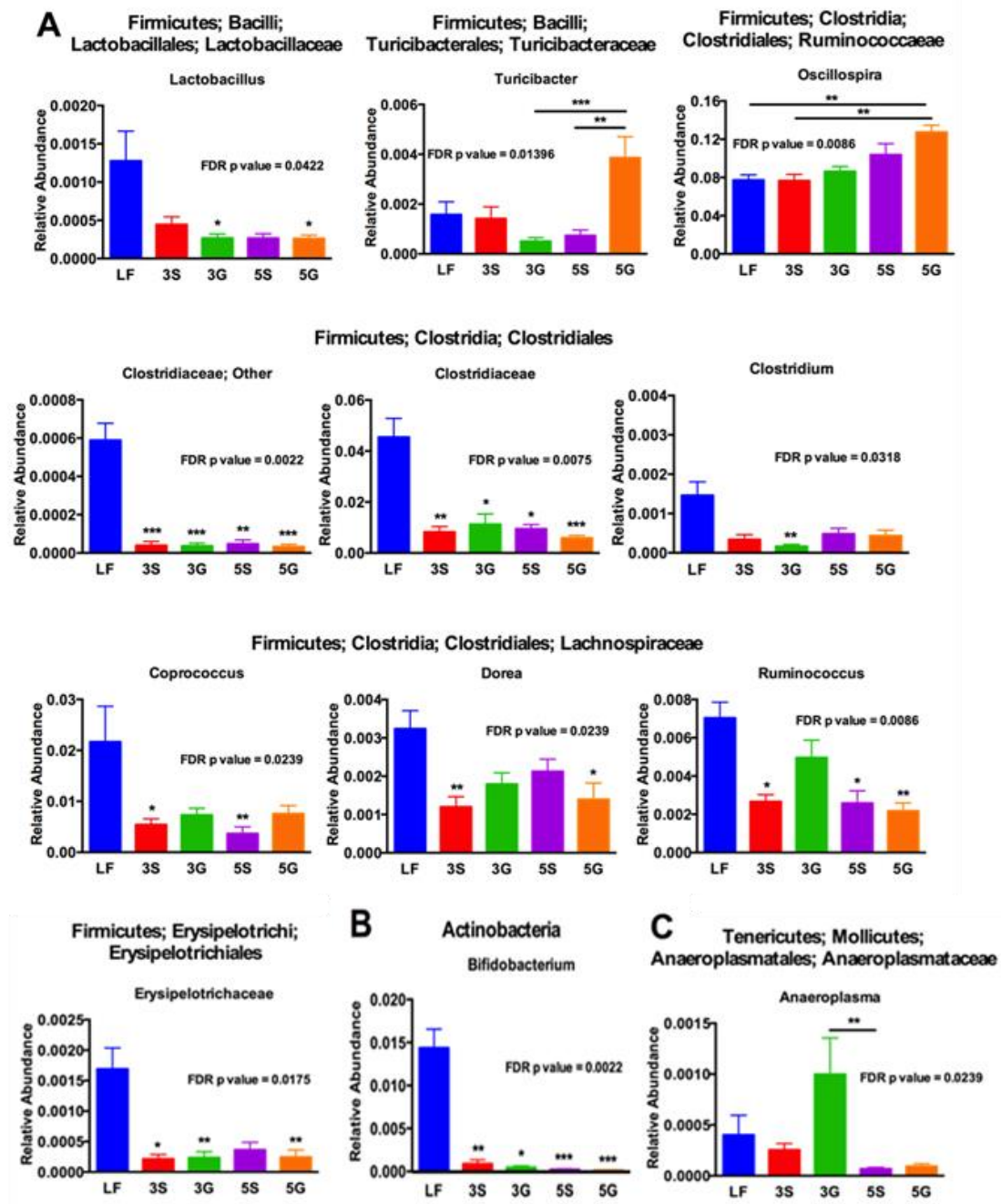


Figure 3.9. Relative Abundance of Microbial Taxa. Significantly altered relative abundances of microbial taxa (i.e., A- Firmicutes, B- Actinobacteria, and C- Tenericutes) found across C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. Taxa shown were significantly altered based on Kruskal Wallis

test run using QIIME software following filtering of OTUs that were not present in 50% of samples. FDR corrected p values based on this analysis are shown. To conduct multiple comparisons relative abundances were analyzed via ANOVA followed by Dunn's test for multiple comparisons. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes. Data are presented as Means \pm SEM (n=9-10). Unless otherwise indicated, asterisks show significant differences of HF diets with or without grapes compared to LF control.

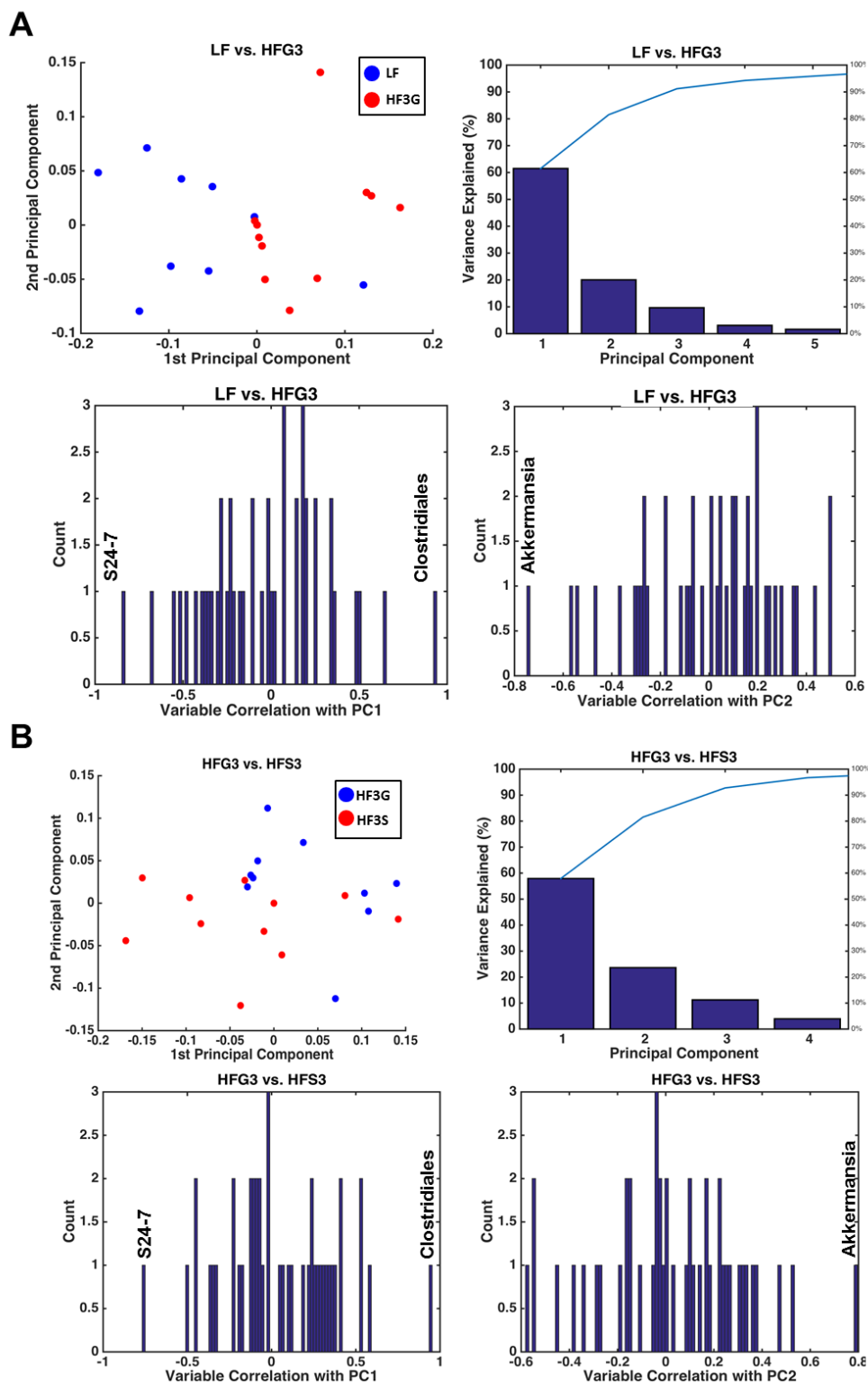


Figure 3.10. Principle Component Analysis (PCA) of Cecum Mucosa. The microbial relative abundances from C57BL/6J mice fed a low fat diet or butter-rich, high fat diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Left - PCA plot between low fat (LF) and high fat + 3% grapes (HF3G) are shown. Middle – Pareto plot showing percentage of variance explained by principal components. Right – Histogram showing individual genera that were negatively or positively correlated with LF and/or HF-3G diets. (B) Same as A but showing PCA of HF-3G vs high fat + 3% sugar (HF-3S) groups.

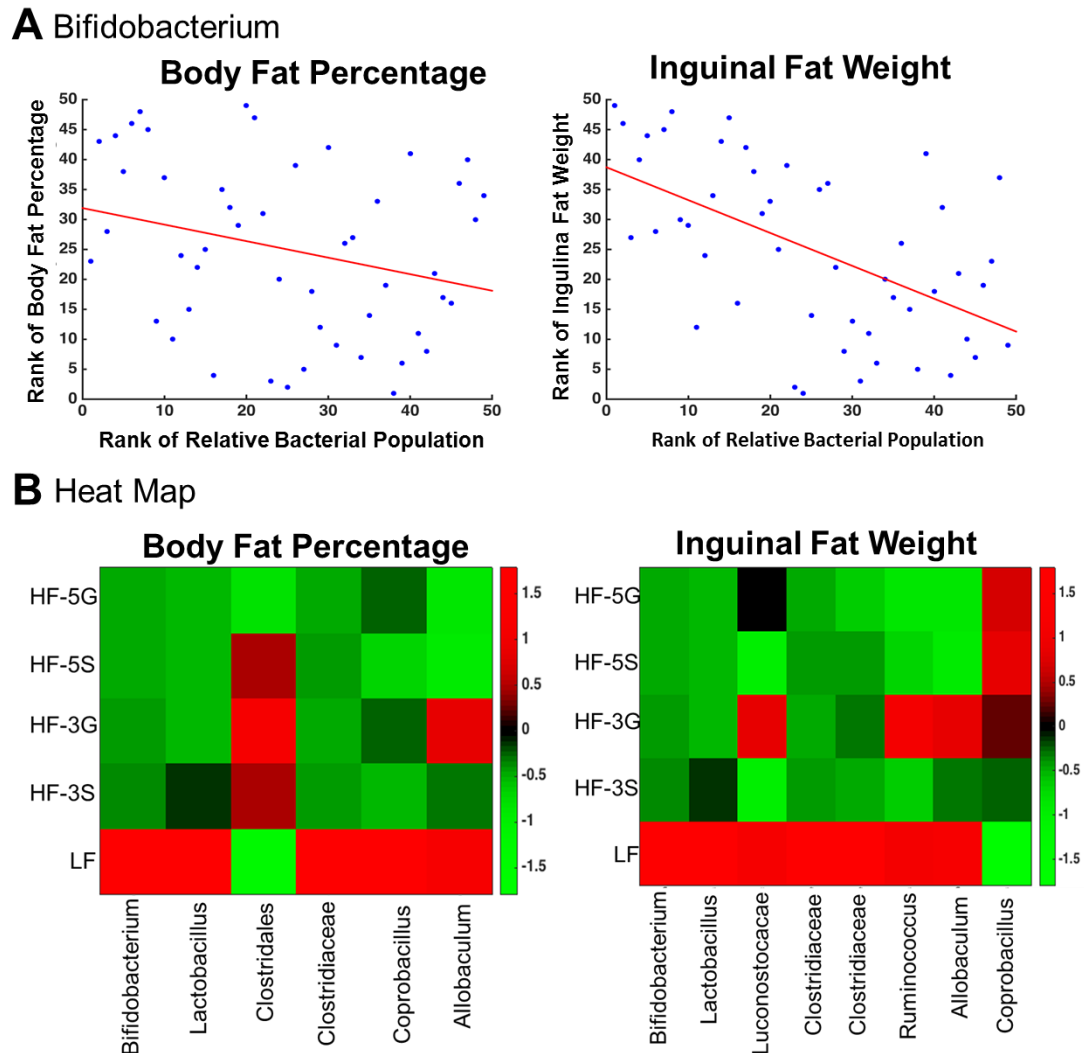


Figure 3.11. Relationship between Body Fat and Bacterial Populations. (A) *Bifidobacterium* relative abundance is negatively correlated with body fat percentage ($r = -0.53$, $p = 0.001$) and inguinal fat pad weight ($r = -0.48$, $p = 0.004$) as determined by Spearman Correlation Analysis. (B) Heatmap showing relative abundances of taxa that were significantly correlated with body fat percentage. LF, low fat; 3S, high fat diet containing 3% sugar; 3G high fat diet containing 3% grapes; 5S, high fat diet containing 5% sugar; 5G, high fat diet containing 5% grapes.

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CHAPTER IV

AN EXTRACTABLE, POLYPHENOL-RICH FRACTION OBTAINED FROM CALIFORNIA TABLE GRAPES DECREASES ADIPOSITY, INSULIN RESISTANCE, AND MARKERS OF INFLAMMATION IN HIGH-FAT FED C57BL/6J MICE

Brian Collins, Jessie Baldwin, Chia-Chi Chuang, Paula Cooney, Robin Hopkins, and Michael McIntosh

Abstract

The objective of this study was to determine the extent to which consuming two methanol-extractable fractions of California table grapes reduced adiposity, hepatic steatosis, or markers of inflammation or lipid metabolism in mice fed a high-fat, American-type diet. Male C57BL/6J mice were fed a low fat diet, a high fat (HF) diet, or a HF diet containing California table grapes (GP; 5% w/w), an extractable polyphenol (HF-EP) fraction from GP, a non-extractable polyphenol (HF-NEP) fraction from GP, or an equal combination of both fractions (HF-EP+NEP) for 16 weeks. Mice fed the HF-EP and HF-EP+NEP-containing diets had lower percentages of body fat and amounts of total white adipose tissue (WAT) and improved glucose tolerance compared to the HF controls. In epididymal WAT, the mRNA levels of the inflammatory genes cluster of differentiation 11c, monocyte chemoattractant protein 1, epidermal growth factor-like module-containing mucin-like hormone receptor 1, tumor necrosis factor alpha, and the lipogenic gene, acylglycerol-3-phosphate-O-acyltransferase 2 were lower in mice fed the HF-EP- and HF-EP+NEP-containing diets compared to HF controls. Mice fed HF-NEP and HF-EP+NEP diets had reduced liver weights and mice fed HF-EP+NEP diets had lower liver triglyceride levels compared to the HF controls. Mice fed the HF-EP+NEP diets had higher hepatic mRNA levels of

hormone sensitive lipase and adipose triglyceride lipase, and decreased expression of C-reactive protein compared to the HF controls. Surprisingly, HF-GP feeding did not decrease adiposity, hepatic steatosis, or inflammation nor did the HF or grape diets alter markers of intestinal inflammation. Taken together, these data demonstrate that the polyphenol-rich EP fraction from California table grapes attenuates many of the adverse health consequences associated with consuming a HF diet, independent of influencing intestinal inflammatory status.

Introduction

Obesity has been on the rise in the U.S. since the 1960's and affects approximately one third of the adult population [1]. The incidence of obesity is also increasing globally, affecting approximately 300 million adults worldwide [2]. Obesity is intimately associated with chronic inflammatory conditions that contribute to the metabolic syndrome (e.g., type 2 diabetes, hypertension, and cardiovascular disease) [3]. Over consumption of calories coinciding with a lack of physical activity are the major risk factors for obesity development resulting in expansion of white adipose tissue (WAT). As WAT grows beyond the capacity of the vasculature system to provide it with nutrients and oxygen, an increase in inflammatory signals recruit macrophages and other immune cells into the WAT. As the immune response progresses, the abundance of circulating and tissue proinflammatory cytokines and chemokines increases [4]. This inflammatory scenario disrupts metabolic processes which results in impaired glucose and fatty acid uptake and metabolism, and hemostasis, thereby contributing to the development of metabolic diseases. Although there is a general consensus on the metabolic consequences of obesity-mediated inflammation, the exact mechanisms that initiate these events are not clearly understood.

Recently, the role that gut microbes play in the development of the metabolic syndrome has received attention due to their sensitivity to environmental changes that can trigger obesity [5],

chronic inflammation [6-8], and insulin resistance [9]. Diets high in fat have been implicated in the reduction of microbial diversity, in particular gut barrier-protecting bacteria as well as increasing the abundance of deleterious bacteria [reviewed in 10]. For example, an increase in the ratio of *Firmicutes* to *Bacteroidetes* is positively correlated with the development of obesity and insulin resistance [11]. Diets rich in saturated fat [11, 12], particularly from milk [13], increase the abundance of sulfidogenic bacteria like *Bilophila wadsworthia* and *Desulfovibrionaceae* spp. which generate hydrogen sulfide gas. Hydrogen sulfide is geno- and cytotoxic and positively correlated with development of ulcerative colitis, gut inflammation, irritable bowel syndrome, and colon cancer [13-15]. Notably, the effects of high fat (HF) diet on body weight gain are repressed in microbiota-free mouse models as well as fecal microbial transplants from healthy donors into obese subjects [16]. Therefore, interventions directed towards modifying the microbial composition of the gastrointestinal (GI) tract may alleviate obesity-mediated outcomes.

The use of non-digestible carbohydrates, fiber, or polyphenols as prebiotics shows promise as potential interventions for the metabolic consequences of obesity [reviewed in 13, 17-19]. Prebiotics are agents that selectively stimulate the growth or activities of specific microbial populations in the gut which translates into health benefits for the host [20]. Fiber, in particular inulin-type fructans, has been shown to increase the abundance of *Bifidobacteria* which was positively correlated with decreased hyperglycemia, endotoxemia, and systemic cytokine levels [21, 22]. Similar effects have been demonstrated in obese subjects with short term supplementation of gluco-oligosaccharides [23]. It is believed that the primary benefit of fiber fermentation by intestinal microbes is through increased productions of short chain fatty acids (SCFA), which have been shown to regulate the synthesis of GI peptides that influence energy intake (i.e., glucagon-like peptide (Glp)-1 and 2, peptide YY (Pyy), and ghrelin) as well as energy

storage and metabolism through interactions with G-protein receptors (Gpr) 41 and 43 [reviewed in 20].

Although much focus has been placed on the beneficial properties of fiber, polyphenols found in fruits and vegetables may also improve GI health [24-27]. Absorption of polyphenols is poor in the upper gastrointestinal tract, leading to increased availability in the lower GI tract [27]. Thus, polyphenols may have a significant influence on microbiota taxa and their metabolites [28]. Indeed, the anti-inflammatory, anti-oxidant, and anti-microbial actions of polyphenols have been reported to positively influence gut microbes and host inflammation [reviewed in 29]. As such, one potential mechanism to improve the outcomes associated with diet-induced obesity is increased consumption of poorly digestible food components including polyphenols.

Grapes and other berries are rich in polyphenols including anthocyanins [reviewed in 29], which have known anti-inflammatory and anti-oxidant effects [30]. These beneficial effects of grapes have been associated with reduced cytokine levels via suppression of nuclear factor kappa B (NF κ B) and increased peroxisome proliferator-activated receptor (PPAR) α/γ [31]. Consistent with these data, we demonstrated that C57BL/6J mice consuming a HF diet (i.e., 60% kcals from lard) supplemented with whole powdered California table grapes (3%, w/w) had improved glucose tolerance after 5 weeks and decreased markers of inflammation ~20-50% in serum and WAT after 18 weeks [32]. We also demonstrated that consuming a moderate fat diet (i.e., 34% kcals from milk fat) supplemented with whole powdered grapes (3% or 5% w/w) reduced adiposity, improved liver triglyceride (TG) levels, modestly reduced WAT inflammatory gene expression, and lowered the cecum levels of sulfidogenic bacteria, while tending to increase the abundance of *Akkermansia muciniphila* and *Allobaculum* in the proximal colon and cecum, respectively (Chapter III).

However, the identities of the bioactive fractions in whole powdered table grapes and the role that gut microbiota play in improvements in diet-induced obesity in mice fed grapes are unknown. Therefore, the objective of this study was to determine the extent to which consuming methanol-extractable fractions of table grapes reduced adiposity, hepatic steatosis, or markers of inflammation or lipid metabolism in mice fed a high-fat, American-type diet. We separated lyophilized powder from whole grapes into two methanol-extractable fractions; i.e., an extractable polyphenol (EP) fraction and a non-extractable polyphenol (NEP) fraction. These fractions were incorporated into a HF, American-type diet (i.e., similar in fat amount and type to the 75th percentile of American diets [33]). This HF diet was fed alone or in combination with the EP fraction, the NEP fraction, both fractions (EP+NEP), or whole grape powder (GP; 5%, w/w) for 16 weeks. Body composition and glucose tolerance were measured at 5 week intervals. At week 16, markers of intestinal and systemic inflammation, insulin resistance, and lipid metabolism were measured.

Materials and Methods

Reagents and materials

Reference compounds procyanidin A2 (PAC-A2), procyanidin B2 (PAC-B2), catechin and epicatechin were purchased from Chromadex (Irvine, CA). 4-dimethylaminocinnamaldehyde (DMAC), Folin-Ciocalteu reagent, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). All organic solvents were HPLC grade and obtained from VWR International (Suwanee, GA).

Extraction and polyphenol enrichment

Freeze-dried grape powder (200 g x 5 batches) was blended each with 1 L 50% acidified methanol (0.1% TFA). The mixture was centrifuged (Sorvall RC-6 plus, Asheville, NC) at 4000

rpm for 10 min, and the supernatant was collected. A sample from the combined supernatants (2 L) was evaporated and freeze-dried to afford the crude extract. The rest of aq-methanol extract was evaporated to remove the organic solvent, and then loaded to Amberlite XDA-7 resin, stirred for 20 minutes, and the supernatant was discarded. The resin was washed with water to get rid of all free sugars and organic acids. The polyphenols were eluted from the resin with 100% methanol, organic solvent was evaporated under vacuum, and the remaining aqueous extract was freeze-dried to afford the EP fraction. The pelleted material (plant debris after extraction) was put in a vacuum oven (45 °C) to get rid of the organic solvent before freeze-drying to afford the NEP fraction.

Alkaline hydrolysis of NEP fraction

Alkaline hydrolysis of NEP fraction was performed according to Yang et al. [34] with some modifications. In 15 mL centrifuge tube, 2.0 mL of 4 M NaOH were added to 0.5 g NEP, flushed with nitrogen, closed and incubated for 1 hour at room temperature. The mixture was adjusted to pH 7 with drops of concentrated hydrochloric acid, then loaded onto a column packed with celite at a ratio 1:2 v/w. The hydrolyzed polyphenols were eluted with 30 mL methanol-ethyl acetate (20:80 v/v), and evaporated to dryness.

Determination of total phenolics, anthocyanins and proanthocyanidins

Total phenolics (TP) were determined in EP and NEP fraction hydrolysates with Folin-Ciocalteu reagent [35]. Concentrations were expressed as mg/L gallic acid equivalents. Total monomeric anthocyanin (ANC) content was measured in EP using the pH differential spectrophotometric method [36], and expressed as cyanidin glucoside equivalent. Total proanthocyanidin content (PAC) was determined in EP using the DMAC method as previously described [37], and quantified as procyanidin A2 equivalent.

HPLC profile analyses of anthocyanins and proanthocyanidins

HPLC analyses were conducted according to the previously reported protocols [38].

Animals

Four-week old, male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and acclimated on a standard chow diet for 1 week. Mice were housed in pairs, maintained at a temperature of 22°C with 50% humidity, and exposed to a 12 h light/ 12 h dark cycle. Mice received food and water *ad libitum* and measures of body weight and food intake were conducted once and twice per week, respectively. All experimental procedures were performed under ethical standards and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Greensboro.

Diets

Animals were randomly assigned to one of six dietary treatments (n=13 per treatment group) as follows: low fat (LF; 10% of energy from fat), HF (45% of energy from fat), HF plus extractable polyphenol fraction (HF-EP), HF plus non-extractable polyphenol fraction (HF-NEP), HF plus extractable and non-extractable polyphenol fraction (HF-EP+NEP) and HF plus 5% powdered grapes (HF-GP) (**Table 4.1**). The HF diets consisted of approximately 10.7% energy from soybean oil, 9.8% of energy from butter, 7.8% energy from lard, 12.2% energy from shortening, and 4.5% energy from beef tallow [33, 39]. Thus, the HF diets were rich in fat with the proportion of the sources of fat mimicking an American-type diet [33]. The amounts of EP and NEP fractions that were added to the HF diets were equal to their relatively amounts in the 5% powdered whole grape diet (i.e., grape powder contains 2.3% and 6.9% EP and NEP, respectively). The lyophilized (i.e., powdered) California table grapes were kindly provided by the California Table Grape Commission and consisted of a mixture of red, green, and purple

seeded and seedless grapes. The 5% dietary level of grapes is comparable to 15 human servings of grapes. Because the extraction process removes the sugar fraction in grapes, a mixture of fructose and glucose was added to the HF, HF-EP, HF-NEP, and HF-EP+NEP diets to control for the sugar content of the powdered grape diet. Detailed composition of the diets is illustrated in Table 4.1.

Intraperitoneal glucose tolerance tests (GTT)s and fasting insulin levels

Intraperitoneal (i.p.) GTTs were performed on weeks 7, 12, and 16 on non-anesthetized mice. Mice were fasted for 8 h and given an i.p. injection of glucose (i.e., 20% solution at 1 g/kg body weight). Blood from the tail vein collected at baseline and 5, 15, 30, 60, and 120 minutes post-i.p. glucose injection was used to quantify glucose levels using a Bayer Contour blood glucose monitor and strips (Bayer Healthcare, Tarrytown, NY, USA). Plasma insulin levels were detected using an ultrasensitive mouse insulin kit (Crystal Chem, Inc, Downers Grove, IL). The homeostasis model assessment method (HOMA) for insulin resistance (IR) was used employing the following formula: $[\text{fasting insulin concentration (ng/ml)} \times 24 \times \text{fasting glucose concentration (mg/dl)}] / 405$ [32].

Body fat measurements via Dual X-Ray Absorptiometry (DEXA)

Percent body fat was measured using DEXA on a GE Lunar Prodigy Advanced System (GE Healthcare, Milwaukee, WI) at weeks 6, 11, and 15. During the measurement, mice were lightly anesthetized with isoflurane using a SomnoSuite Small Animal Anesthesia System with Integrated Digital Vaporizer isoflurane system. Measurements were taken in duplicate or triplicate to reduce the possibility of error and values expressed are an average of the two measurements.

Tissue collection

After 16 weeks of dietary intervention, mice were fasted for 8 h and euthanized via isoflurane-induced anesthesia followed by cardiac puncture. Plasma was collected at the time of harvest. Four WAT depots were collected; epididymal, mesenteric, inguinal, and retroperitoneal. Additionally, livers were harvested and intestinal mucosa and digesta were collected from the duodenum, jejunum, ileum, cecum, or proximal or distal colon. Weights of the WAT depots and liver were recorded and all collected samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Liver and plasma TG levels

Liver TG content was measured as previously described [40]. Plasma TG content was determined using a commercial assay from Thermo Scientific and was conducted following the manufacturer's protocol (Infinity TG assay #TR22421 and TG standards #TG22923; Norcross, GA).

Liver Oil-Red-O Staining

Liver tissues were frozen in OCT compounds, cut at 5 µm, and mounted on slides. The sections were fixed with 10% formalin for 10 minutes and then the slides were washed with deionized water for 5 minutes. Fixed tissues were then rinsed with 60% isopropanol for 5 minutes. To prepare Oil red O stock, 0.5 g of Oil red O (Sigma-Aldrich) was mixed with 100 mL of isopropanol. To prepare Oil red O working solution, 30 mL of Oil red O stock was mixed with 20 mL of distilled water and filtered using a 0.24 µm vacuum filter. Samples were submerged in the working solution for 15 minutes, briefly rinsed with 60% isopropanol, and then rinsed with deionized water for 30 seconds before imaging.

RNA extraction and qPCR

Adipose and intestinal samples were homogenized in QIASol reagent and total RNA was extracted using QIAgen mini lipid kit obtained from Qiagen (Valencia, CA). For hepatic samples, a QIAgen mini universal kit from Qiagen was used. The quality and concentration of RNA were examined using absorbance at 260 nm and integrity determined using the absorbance ratio of 260/280 on a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA was created by reverse transcription using 1 ug of RNA and a high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocols. qPCR was performed in a 7500 FAST Real Time PCR system (Applied Biosystems). The expression of different genes related to inflammation, lipogenesis, and lipolysis in WAT depots and liver were measured using Taqman Gene expression assays purchased from Applied Biosystems. TATA-binding protein (*Tbp*) was the endogenous reference gene utilized for all assays and fold differences in gene expression were calculated as $2^{-\Delta\Delta C_t}$.

Statistical Analysis

Data were analyzed using a one-way ANOVA and Student's *t* tests to compute individual pairwise comparisons of means ($p < 0.05$). Bonferroni's posthoc tests were also performed for specific comparisons where appropriate. Analyses were conducted using the JMP software program version 10.0 for Windows (SAS, Cary, NC). Data are expressed as means + S.E.M.

Results

Polyphenol content and profile of EP, NEP, and GP

Polyphenol analysis revealed that the weight percentages of polyphenols in the EP and NEP fractions were 2.26% and 6.91% respectively. The EP fraction contained 180.0 mg/g of total phenolics and the NEP fraction contained 10.5 mg/g of total phenolics. Total anthocyanin and

proanthocyanidin content of the EP fraction was 37.8 mg/g and 305.5 mg/g, respectively, whereas these polyphenols were not detectable in the NEP fraction (**Table 4.2**). Individual anthocyanin analysis revealed that the most abundant anthocyanins present in the EP fraction were peonidin-3-O-glucoside (36.6 u/mg), malvidin-3-O-glucoside (30.8 u/mg), and peonidin-3-O-cis (6''-O-p-coumaryl)-glucoside (28.5 u/mg) (**Table 4.3**). The profile of the degree of polymerization of the proanthocyanidins in the EP fraction is shown in (**Fig. 4.1**).

EP fraction with or without NEP fraction lowers body fat gain

Body weight gains and energy intakes were greater in all HF-fed mice compared to the LF control mice (**Table 4.4**). There was no difference in the energy intake between all of the HF-fed mice, with the exception of the HF-EP+NEP group that consumed approximately 10% fewer calories than the HF controls (Table 4.4). Mice fed the HF-EP and HF-EP+NEP diets had lower body weight gains (Table 4.4; ~28% and 40%, respectively) and body fat percentages than the HF controls (**Fig. 4.2**; 27% and 37%, respectively). Mice fed the HF-EP, HF-NEP and HF-EP+NEP diets had lower total WAT depot weights compared to HF controls (Fig.4.2). Paradoxically, mice fed the HF-GP diet did not have improvements in body fat percentage, body weight, and total WAT depot weights compared to the HF controls.

EP fraction improves glucose disposal, insulin resistance, and hypertriglyceridemia

To assess the impact of grape consumption on glucose disposal and insulin resistance, GTTs were conducted at weeks 7, 12, and 16, and fasting plasma insulin and HOMA-IR were measured at week 16, respectively. Mice consuming the HF control diet had impaired GTTs at all three time points compared to the LF controls (**Fig. 4.3A**). This HF diet impairment was attenuated at all three time points in mice fed the HF-EP diet. Mice fed the HF-NEP and HF-EP+NEP diets had improved GTTs at weeks 12 and 16 compared to the HF controls. HOMA-IR index calculations

at week 16 indicated that mice consuming the HF-EP+NEP fraction had improved insulin sensitivity compared to the HF controls (**Fig. 4.3B**). Surprisingly, consuming grapes did not significantly improve GTT, fasting plasma insulin, and HOMA-IR measurements. Mice fed the HF-EP and the HF-EP+NEP diets had lower plasma TG levels compared to the HF-fed controls (**Fig. 4.3C**).

Grape fraction consumption lowers hepatic TG levels and alters the expression of genes associated with lipid metabolism and inflammation

Liver tissue was analyzed to assess the impact of the diets on liver TG levels and markers of lipogenesis, lipolysis, and fatty acid oxidation. Mice consuming the HF control diet had greater liver weights, TG content, and mRNA levels of the lipogenic genes *Ppar γ* and *Fas* compared to the LF-fed mice (**Fig. 4.4**). Compared to LF controls, HF feeding also increased gene expression of stearyl regulatory element binding protein 1 (*Srebp-1c*), and stearyl-CoA desaturase 1 (*Scd1*), which was not effected by whole grape or grape fraction feeding (Figure 4.4). Mice consuming the HF-NEP and HF-EP+NEP diets had lower liver weights and mice consuming the HF-EP+NEP diet had lower liver TG content compared to HF controls. Mice fed the HF-EP and HF-EP+NEP diets had greater mRNA levels of hormone sensitive lipase (*Hsl*), while mice fed the HF-EP+NEP diet had increased mRNA levels of adipose triglyceride lipase (*Atgl*) compared to HF controls (Figure 4.4). HF feeding increased the hepatic gene expression of the inflammatory marker C-reactive protein 1 (*Crp1*), which was attenuated by HF-EP and HF-EP+NEP feeding (Figure 4.4). Interestingly, all mice consuming HF diets had ~60% lower mRNA levels of phosphoenolpyruvate carboxykinase (*Pck1*), a marker of gluconeogenesis. No treatment differences in the expression of carnitine palmitoyltransferase 1-a (*Cpt1a*) were detected (data not shown). Hepatic oil-red-o staining indicated greater lipid content in the livers of mice fed the HF

control diet compared to the LF controls, which was attenuated by HF-EP, HF-NEP, and HF-EP+NEP consumption (**Fig. 4.5**).

EP fraction with or without NEP fraction improves the expression of WAT genes associated with inflammation and lipid metabolism

To determine if the negative metabolic effects caused by HF-feeding were associated with inflammation in WAT, mRNA levels of several proinflammatory genes were measured in inguinal (subcutaneous) and epididymal (visceral) WAT. In inguinal WAT, mice fed the HF control diet had greater mRNA levels of cluster of differentiation 11c (*Cd11c*) and monocyte chemoattractant protein 1 (*Mcp1*) compared to LF controls (**Fig.4.6A**). This HF-induced increase was attenuated in mice fed the HF-EP and HF-EP+NEP diets. In epididymal WAT, HF-feeding increased mRNA levels of *Cd11c*, *Mcp1*, tumor necrosis factor alpha (*TNFA*), and epidermal growth factor-like module containing mucin-like hormone receptor 1 (*Erm1*; F4/80 human orthologue) compared to LF controls (**Fig. 4.6B**). Consistent with the inguinal data, mice fed the HF-EP and HF-EP+NEP diets had lower mRNA levels of these genes compared to the HF controls.

To determine whether the reduction in adiposity observed with feeding of HF-EP, HF-NEP, and HF-EP+NEP diets was associated in alterations in lipid metabolism, inguinal and epididymal WAT were examined for markers of; (i) lipogenesis (i.e., *Pparγ*, *Srebp1c*, *Scd1*, acylglycerol-3-phosphate-O-acyltransferase 2 (*Agpat2*), fatty acid synthase (*Fas*), glycerol-3-phosphate acyltransferase2 (*Gpat2*)), (ii) lipolysis (i.e., *Atgl*) (iii) beta-oxidation (i.e., carnitine palmitoyltransferase 1-b (*Cpt1b*), *Ppara*), (iv) SCFA receptors (i.e. *GPR43*), and (v) plasminogen activator inhibitor-1 (*Pai-1*). In inguinal WAT, mice fed the HF diet had greater expression levels of *Agpat2*, which were lower in the HF-EP and HF-EP+NEP groups (**Fig. 4.7A**). *Cpt1b* gene

expression was lower in mice fed the HF-EP, HF-EP+NEP diets compared to the HF-fed mice. No treatment differences in gene expression were observed for *Srebp1c*, *Scd1*, *Fas*, *Gpat2*, *Atgl*, and *Ppara*.

In epididymal WAT, mice consuming the HF diet had higher mRNA levels of *Agpat2*, a marker of TG biosynthesis, compared to LF controls (**Fig. 4.7B**). Feeding HF-EP and HF-EP+NEP attenuated this HF-induced increase in *Agpat2* expression. Additionally, mice fed the HF diet had lower levels of *Ppar γ* compared to LF controls. The expression of *Ppara*, a marker of beta oxidation, was reduced in mice fed the HF control and the GP diets compared to LF controls. Surprisingly, HF feeding increase the mRNA levels of *Cpt1b*, beta-oxidation marker, which was reduced by HF-EP and HF-EP+NEP feeding. Additionally, mice fed the HF-EP+NEP diet had lower mRNA levels of *GPR43*, a SCFA-activated receptor associated with increased energy harvest, compared to HF- and LF-fed controls. No differences in gene expression in epididymal WAT were observed for *Srebp-1c*, *Scd1*, *Fas*, *Gpat2*, and *Atgl* (data not shown).

Minimal influence of HF-feeding and grape fraction consumption on markers of intestinal inflammation

Given the reported adverse effects of consuming saturated fats [reviewed in 10] on intestinal health, and potential prebiotic impact of grapes, we measured the effects of the diets on markers of intestinal inflammation and barrier function. For inflammatory status, the mRNA levels of *Cd11c*, *Cd68*, *Erml1*, *Mcp1*, *Tnfa*, toll-like receptor 4 (*Tlr4*), and interleukin (*Il*)*1b* in ileum and proximal colon mucosa and the activity of ileal myeloperoxidase were measured. Unexpectedly, the mRNA levels of proinflammatory genes were not increased in the ileal mucosa of HF-fed mice compared to the LF controls. Equally surprising, mice consuming the HF-GP diets had greater mRNA levels of *Tlr4* and *Cd68* compared to the HF-fed mice (**Fig. 4.8A**). Although the

activity of myeloperoxidase, an enzyme expressed neutrophils and indicative of neutrophil infiltration, was ~50% lower in the ileal mucosa of mice consuming the HF-EP and HF-EF+NEP diets compared to the HF-fed mice, these differences were not statistically significant (**Fig. 4.8A**). Similarly, the expression of *Tlr4* was greater in the colonic mucosa of HF- GP fed mice compared to the HF controls (**Fig. 4.8B**). Two of the proinflammatory genes measured in the colonic mucosa were increased by HF feeding (i.e., *Cd11c*, *Cd68*) and only *Cd68* was attenuated in mice consuming the HF-EP+NEP diet (Fig. 4.8B).

Influence of grape fractions on intestinal L-cell gene expression

Intestinal microbial metabolites impact local and systemic health, in part, by influencing fermentation products (e.g., SCFA), energy harvest, and the release of endocrine signals from L-cells located in the mucosa epithelium. Locally, these metabolites influence the integrity of the mucosa barrier and systemically impact energy balance and carbohydrate and lipid metabolism [20]. Therefore, we determined the influence of the dietary treatments on the induction of genes associated with SCFA receptors (e.g., *Gpr41*, *-43*, and *-119*) and two of their downstream targets (e.g., *Glp* and *Pyy*) in the ileum and proximal colon. Mice fed the HF-EP, HF-NEP, and HF-EP+NEP diets had greater mRNA levels *Gpr43*, but not *Gpr41*, in the ileum mucosa compared to the HF controls (**Fig. 4.9A**). Mice fed the HF-EP+NEP and the HF-GP diets had lower mRNA levels of *Gpr41* in the ileum mucosa compared to the HF controls. Mice fed the HF-GP diet has the highest levels of *Glp* compared to all other treatments (Fig.4.9A). In contrast, mice fed the HF diet had higher mRNA levels of *Gpr43* in the proximal colon compared to the HF-NEP, HF-EP+NEP, and HF-GP groups (**Fig. 4.9B**). Lastly, HF-feeding increased *Pyy* mRNA levels in the proximal colon, which were lower in mice fed the HF-EP+NEP diet.

Discussion

Rationale and significance of this study

We previously demonstrated that feeding whole GP in conjunction with a HF diet (i.e., 34% kcals from fat; 3% from soybean oil and 31% from milk fat, which was similar to the amount of fat consumed by Americans in the 50th percentile [33] for 10 weeks modestly reduced adiposity (3% and 5% GP), hepatic steatosis (5% GP), and the ileal mucosa abundance of the deleterious sulfidogenic bacterium species *DSB* and a sulfidogenic gene *drsA-Bw* (3% GP), and increased the proximal colon mucosa abundance of the beneficial bacterium *Akkermansia muciniphila* (5% GP) and the localization of the tight junction protein ZO-1 on the apical epithelial surface of the ileum (3% and 5% GP). However, consuming the HF diet for 10 weeks only modestly increased adiposity, insulin resistance, and several markers of inflammation in epididymal (visceral) WAT, and had no effects on markers of intestinal inflammation. Therefore, we conducted the current 16 week study using a higher level of dietary fat (i.e., 45% kcals) and a fatty acid profile that was similar to Americans in the 75th percentile (i.e., soybean oil, butter, lard, shortening, and beef tallow [33] to enhance the metabolic consequences of diet-induced obesity. We also wanted to know which fraction of GP was responsible for reducing adiposity and its potential metabolic complications. Therefore, we examined the effects of whole GP (i.e., 5%) and two isolated fractions of grapes (i.e. methanol derived EP and NEP fractions which were fed at amounts equal to those in 5% GP) on the development of obesity, steatosis, glucose intolerance, and WAT inflammation in C57BL/6J mice fed a HF diet similar to Americans. The EP fraction contained ~16 times more polyphenols, particularly anthocyanins (i.e., glucosides of anthocyanidins; Table 4.3) and proanthocyanidins (i.e., polymers of anthocyanidins formed by the condensation of flavans; Table 4.2), than the NEP fraction. The results indicate that in the context of HF feeding, consumption of the polyphenol-rich, EP fraction, alone or in combination with the NEP fraction,

attenuated diet-induced obesity, insulin resistance, steatosis, and chronic inflammation in WAT. However, consuming whole GP had no beneficial impact on the outcomes measured, and reasons for this lack of an effect are unclear.

Potential mechanisms by which anthocyanin-rich EP fraction suppresses adiposity and inflammation and improves insulin sensitivity

Anthocyanins and proanthocyanidins are groups of flavonoids present in relatively large amounts in table grapes. Anthocyanins give these berries their unique colors. Our phenolic analysis indicated that the most abundant anthocyanins found in California table grapes are malvidin-3-O-glucoside, peonidin-3-O-glucoside, and cyanidin-3-O-glucoside. While studies have demonstrated anti-obesity and anti-diabetic effects of anthocyanins [reviewed in 41], studies focusing on the effects of feeding whole grapes and grape fractions feeding in subjects consuming a diet similar in fat amount and types to the American diet are lacking. Consistent with our anthocyanidin-rich EP data, mice consuming a HF diet (i.e., 60% kcals from fat, primarily lard) supplemented with anthocyanin-rich muscadine grape or wine extracts for 15 weeks had lower body weights, plasma glucose and TG levels, insulin resistance, and CRP levels compared to controls (42). Also, dietary supplementation of grape seed procyanidins has been demonstrated to prevent body weight gain, reduces WAT inflammatory markers (i.e. *Tnfa*, *Il-6*, *Crp*) in high-fat-fed rats (43). Similar effects were observed with grape seed extract supplementation including reductions in weight gain, WAT weights and blood lipid levels in HF fed mice (44), and improvements insulin sensitivity and reductions in oxidative stress in high-fructose fed rats (45). These findings are consistent with our data demonstrating that EP-fed mice had less body weight gain, reduced WAT weights, lower plasma TG, improved insulin sensitivity, and reduced markers of inflammation in WAT (i.e. *Cd11c*, *Mcp1*, *F4/80*).

While research is lacking on the effects of grape anthocyanins specifically, anthocyanins from other dietary sources have been well documented in the literature. Anthocyanins have been demonstrated to consistently exert anti-inflammatory and anti-obesity effects in vitro, and in some instances in vivo. For example, anthocyanins from black soybeans reversed weight gain, reduced the levels of serum TG and cholesterol, and increased the levels of high-density lipoprotein (HDL) (46). These findings are consistent with our data demonstrating that mice fed the anthocyanin-rich EP fraction had reduced body weight gain and plasma TG levels compared to the HF controls. Also, anthocyanins from purple corn prevented HF diet induced increases in body weight and adipose tissue weights in mice (47). Similarly, in HF (60% kcals from lard) fed mice, purified anthocyanins from blueberries prevented body weight gain and fat gain compared to controls (48). Additionally, tart cherries, similar to grapes with respect to anthocyanin profile, reduced fat mass and WAT markers of inflammation including Il-6 and Tnfa. (49). These data are consistent with our data demonstrating that the anthocyanin-rich EP fraction decreased body weight gain, fat mass, plasma TG levels, and WAT expression of inflammatory markers including *Tnfa*, *Mcp1*, *Cd11c*, *F4/80*. Additionally, 3T3-L1 preadipocytes treated with anthocyanins had decreased TG accumulation and gene and protein expression of *Pparγ* and *Fas* (50). While we did not observe alterations in expression of *Pparγ* and *Fas* within the WAT, we did observe that mice fed the EP fraction had reduced expression of the lipogenic gene *Agpat2*.

We also demonstrated in this study that mice fed the anthocyanin-rich EP fraction had improved glucose tolerance and insulin sensitivity compared to the HF controls. Similar effects of improvements in glucose tolerance were observed with supplementation of anthocyanins from Maqui Berry in HF fed mice (51) and also with supplementation of anthocyanins from Cornelian cherry in HF fed mice. It has been reported that cyanidin-3-O-glucoside supplementation improves insulin sensitivity in diabetic mice via down-regulation of retinol binding protein and

up-regulation of GLUT4 gene expression (53). This could be a potential mechanism by which the EP fraction improved glucose tolerance and insulin sensitivity.

One novel finding of our study was that the anthocyanin-rich EP fraction increased expression of *Hsl*, a gene associated with lipolysis, and reduced expression the lipogenic-related gene, *Ppar γ* within the liver. This indicates that the EP fraction may reduce steatosis through up-regulation of lipolytic pathways and suppression of lipogenic pathways. Further research examining the effect of the EP fraction on these pathways would be beneficial to better understand the mechanisms by which the EP fraction reduces adiposity or steatosis.

Relationship of intestinal inflammation to systemic inflammation

High fat feeding is known to increase body fat mass and has been shown to consistently induce chronic, low grade inflammation systemically [reviewed in [54]. However, the link between diet and intestinal inflammation has not been as extensively studied. In the current study, HF feeding did not increase markers of inflammation in the ileum or proximal colon after 16 weeks, nor was there any beneficial effect of GP or grape fraction consumption. However, other research has shown that HF diets can trigger intestinal inflammation or endotoxemia that may increase systemic inflammation, possibly due to intestinal dysbiosis and barrier dysfunction [7-13]. For example, mice fed a HF diet had increased levels of saturated fatty acids and lipopolysaccharide (LPS) within intestinal tissue samples resulting in increased *Tnf- α* and *Il-6* expression through activation of the toll-like receptor Tlr4/Nf- κ B signaling pathway [18]. Mice fed a HF diet were also more susceptible to dextran sulfate sodium (DSS) induced colitis [19]. As a result the immune response was reduced which was associated with an increase in non-CD1d-restricted natural killer T (NK T) cells, which produce inflammatory cytokines similar to those secreted by adipocytes, decreasing the number of regular NK T cells normally found in the

colon. In addition, dietary supplementation with bioactive food components such as grape seed extract and red wine extracts has been shown to improve the intestinal health of subjects fed HF diets. For example, chronic consumption of grape seed extract increases tight junction protein and reduces fecal calprotectin; a neutrophil protein used as a marker of intestinal inflammation [55], and attenuates inflammatory signaling in chemically-induced colitis [56]. Furthermore, in Wistar rats treated with concentrated grape juice, induced colitis was attenuated [57]. In the current study, consumption of the EP and EP+NEP fractions notably attenuated several proinflammatory markers in the WAT, but only EP+NEP decreased the expression of the macrophage markers *Cd68* in the proximal colon. Although the activity of the neutrophil enzyme myeloperoxidase was decreased by 40-50% in the EP and EP+NEP groups compared to the HF controls, these differences were not statistically significant. However, measuring the protein levels of inflammatory markers and the activity of myeloperoxidase and alkaline phosphates in the colon may provide more insight. Alternatively, the intestinal linkage to systemic effects of HF and grape fraction feeding may be due to changes in gut microbiota, as indicated in our first study (Chapter III).

The intestinal microbiota is highly influenced by environmental changes which have been linked to alterations in host health and energy intake. Research has shown that alteration to the gut microbiota by HF feeding can result in increased adiposity, insulin resistance and increased inflammatory cytokines and chemokines [58 -60]. This has been linked to impaired gut barrier function (i.e., the inhibition of tight junction protein production or reduction of mucin secretion from goblet cells) leading to endotoxemia [61,62] and impaired regulation of energy intake and metabolism through activation TLR-dependent enteroendocrine signaling pathways resulting in impaired neural response to leptin [reviewed in 63]. Conversely, microbial consumption of dietary fiber and polyphenols such as those found in grapes, may reduce intestinal and systemic

inflammation through regulation of energy intake and improved intestinal barrier function [reviewed in 64]. Grape polyphenols in particular may have a major impact on the microflora of the large intestine. For example, red wine grape polyphenols given to humans for 4 weeks significantly increased the number of *Enterococcus*, *Prevotella*, *Bacterioidetes*, and *Bifidobacterium* bacteria which correlated to improved blood pressure, and serum levels of TG, total cholesterol, and CRP [65]. Further evidence of the prebiotic effects of grape consumption can be seen in rats given grape pomace juice which increased fecal counts of *Lactobacillus* and *Bifidobacterium* which consequently also resulted in an increase in the concentration of primary bile acids, cholesterol, and cholesterol metabolites while decreasing the concentration of secondary bile acids [66]. This suggests that alteration of the microflora by grape polyphenols may inhibit cholesterol absorption thus improving circulating levels. Indeed, fermentation of fiber and polyphenols by colonic microbes into SCFA has been shown to regulate energy intake and metabolism through stimulating the release of glycoproteins (e.g., Glp-1, Glp-2, and Pyy) by activating Gpr43 and Gpr41 on enteroendocrine cells [reviewed in 67]. Butyrate in particular is also beneficial to intestinal homeostasis as an endothelial energy source as well as through increasing tight junction protein synthesis and mucin secretions [68, 69]. In the current study, the expression of several markers of enteroendocrine cell secretions associated with SCFA activation were upregulated. However, these results were conflicting between the ileum (i.e., *Gpr43* mRNA levels increased by the EP, NEP, EP+NEP diets) and the proximal colon (i.e., *Gpr43* mRNA levels increased by HF diet only). Future analysis of the intestinal microbiota composition, protein levels of Gprs, Pyy, and Glp1/2 levels, and gut barrier function should provide more insight about the extent to consuming HP, GP, and grape fractions influences these intestinal biomarkers.

Limitations and unanswered questions

The beneficial systemic effects of consuming the EP grape fraction may be due to its rich anthocyanidin content. However, the whole GP diet did not have the same positive outcomes as the EP or NEP diets. There were several differences in the design of our previous (Chapter III) and current study that may have contributed to these conflicting results. The previous research was shorter (10 weeks) compared to the current study (16 weeks) and the diet composition with regards to fat (i.e., primarily butter fat at 34% kcals versus a combination of butter, lard, shortening, and beef tallow at 45% kcals from fat, respectively) and fiber (50 g versus 40 g cellulose, respectively). The additional 6 weeks in length of the current study may have caused any potential health benefits of the whole grape diet to have diminished. Yet, by week 10 of the second study, there were no differences between the HF and GP group in regards to body weight, body fat percentage, and glucose tolerance. Furthermore, there may have been a different interaction between the GP and the four types of fats in the current study versus the sole fat source (butter) in the first study (i.e., the polyphenols in the whole GP may interact with butter differently than with the other fat sources, thereby affecting their bioaccessibility and bioavailability). Indeed, the fat content of the diet can differentially impact polyphenol digestion and absorption [70]. Such an interaction may have enabled the polyphenols to inhibit lipase activity, possibly affecting the rate of fat absorption. The increase in calories in the current study may have been too excessive for the beneficial effects of the whole GP to have a positive impact on lipid absorption as well. In order to increase the percentage of fat in the current study, the amount of cellulose and corn starch were reduced compared to the previous study. The alteration in carbohydrate and fiber content may have resulted more time for increased absorption of macronutrients. Such a scenario would result in greater energy harvest, adipogenesis, and triglyceride synthesis in the liver and WAT, independent of any effects by the grape powder.

Furthermore, the extraction process of the anthocyanin rich fraction may have increased the bioaccessibility and subsequent bioavailability of the polyphenols within the powder by liberating them from interactions within the food matrix, making them more susceptible to interactions with brush boarder enzymes potential enhancing their absorption in the small intestine. Similarly, liberation of the polyphenols may have altered the luminal environment, thereby improving the confluence of beneficial bacteria.

Another possible impact of the reduced fiber may have been on the microbiota of the large intestine, as fiber is a primary energy source for a vast majority of microbes in this environment. As such, the reduced fiber may impact the production of SCFAs as well as reduce the diversity of the microbiota, which typically results in intestinal inflammation, impaired barrier function, or adverse systemic effects. Interestingly, although the GP diet had no positive health impact in the second study, the extractable polyphenol fraction did have a positive effect on reducing HF-mediated adiposity and systemic inflammation. Future studies should focus on feeding single anthocyanins and there combinations (i.e., candidates in Table 4.3) in conjunction to a HF diet to see which may be the most effective.

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plasma insulin using an ELISA assay. Mary-Ann Lila and Mary Grace together performed the methylated extraction process to produce the EP and NEP fraction and measured the concentration and composition of the polyphenols present. Chia-Chi Chuang measured liver triglyceride levels and Oil-Red-O liver staining. Paula Cooney measured the body fat mass using DEXA (with the assistance of Brian Collins and Jessie Baldwin).

Table 4.1. Diet Formulation

Ingredients (gram)	LF	HF	GP	EP	NEP	EP+NEP
Casein	200	200	200	200	200	200
L-Cystine	3	3	3	3	3	3
Corn Starch	306.2	73.2	73.2	73.2	73.2	73.2
Maltodextrin 10	125	94	94	94	94	94
Fructose	0	21.9	0	21.9	21.9	21.9
Dextrose, Monohydrate	0	21.9	0	21.9	21.9	21.9
Sucrose	68.8	139	139	139	139	139
Cellulose	50	40	40	40	40	40
Soybean Oil	25	48.8	48.8	48.8	48.8	48.8
Butter	0	44.7	44.7	44.7	44.7	44.7
Lard	20	35.3	35.3	35.3	35.3	35.3
Shortening	0	55.6	55.6	55.6	55.6	55.6
Beef Tallow	0	20.3	20.3	20.3	20.3	20.3
Mineral Mix	10	10	10	10	10	10
DiCalcium Phosphate	13	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5	5.5
Potassium Citrate	16.5	16.5	16.5	16.5	16.5	16.5
Vitamin Mix	10	10	10	10	10	10
Choline Bitartrate	2	2	2	2	2	2
NEP Grape Fraction	0	0	0	0	2.97	2.97
EP Grape Fraction	0	0	0	0.97	0	0.97
Grape Powder	0	0	43	0	0	0
Total	1055.1	854.7	857.5	855.7	857.7	858.7

*LF, low fat control; HF, high fat sugar control; GP, grape powder; EP, extractable polyphenol fraction; NEP, non-extractable polyphenol fraction.

Table 4.2. Phenolic Composition of Grape Powder (GP), Extractable Polyphenol (EP) Fraction and Non-extractable Polyphenol (NEP) Fraction

Assay	GP	EP	NEP
Weight percentage (%)	100	2.26	6.91
Total phenolics ¹	9.13	180.0 ± 1.3	10.5 ± 1.02
Total anthocyanins ²	0.33	37.8 ± 1.8	ND
Total proanthocyanidins ³ (mg/g)	3.05	305.5 ± 7.0	ND

Total phenolics, mg/g DM as gallic acid equivalent (Folin Ciocalteu assay)

²Total anthocyanins, mg/g DM as cyaniding glucoside equivalent (pH differential assay)

³Total proanthocyanidins, mg/g DM as cyaniding glucoside equivalent (DMAC assay)

Table 4.3. Anthocyanin Concentration in Grape Powder (GP) and the Extractable Polyphenol (EP) Fraction

Peak #	Identification	GP Concentrations (mg/kg)	EP Concentrations (μ/mg)
1	Delphinidin-3- <i>O</i> -glucoside		4.93
2	Pyranidin-3- <i>O</i> -glucoside		10.56
3	Petunidin-3- <i>O</i> -glucoside		4.91
4	Peonidin-3- <i>O</i> -glucoside		36.56
5	Malvidin-3- <i>O</i> -glucoside	145.2	30.83
6	Malvidin-3- <i>O</i> -(6"- <i>O</i> -acetyl-glucoside)		3.70
7	Petunidin-3- <i>O</i> -cis-(6"- <i>p</i> -coumaryl-glucoside)		2.56
8	Cyanidin-3- <i>O</i> -(6"- <i>O</i> - <i>p</i> -coumaryl-glucoside)	125.0	2.21
9	Petunidin-3- <i>O</i> -trans-(6"- <i>O</i> - <i>p</i> -coumaryl-glucoside)		3.24
10	Peonidin-3- <i>O</i> -cis-(6"- <i>p</i> -coumaryl-glucoside)	31.7	2.73
11	Malvidin-3- <i>O</i> -cis(6"- <i>p</i> -coumaryl-glucoside)		2.83
12	Peonidin-3- <i>O</i> -cis-(6"- <i>O</i> - <i>p</i> -coumaryl-glucoside)		28.460
13	Malvidin-3- <i>O</i> -trans-(6"- <i>O</i> - <i>p</i> -coumaryl-glucoside)		4.93
	Total anthocyanins		133.52

Table 4.4. Body Weight Gain (BWG), Food Conversion Efficiency (FCE), and Total Calorie Intake per Treatment Group.*

Diets	BWG (g)	FCE (per cage, g food/g BW)	Kcal Intake (per cage)
LF	11.4±0.5 ^d	28.74±1.24 ^a	2502.26±82.01 ^c
HF	24.8±1.0 ^a	13.71±0.35 ^{cd}	3253.91±99.43 ^a
EP	17.8±0.9 ^b	18.43±0.46 ^b	3135.50±165.40 ^{ab}
NEP	20.2±0.9 ^b	15.98±0.63 ^c	3064.54±121.38 ^{ab}
EP+NEP	15.0±0.9 ^c	20.71±1.21 ^b	2914.92±63.30 ^b
GP	24.9±1.0 ^a	13.26±0.31 ^d	3144.70±97.74 ^a

*LF, low fat control; HF, high fat sugar control; GP, grape powder; EP, extractable polyphenol fraction; NEP, non-extractable polyphenol fraction.

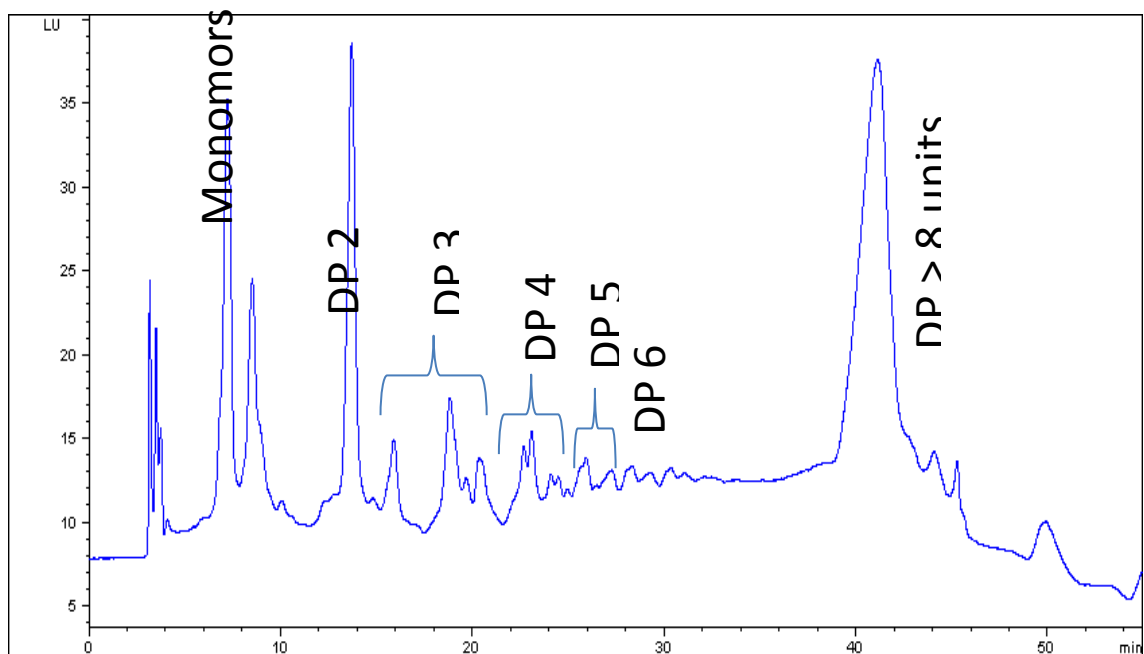


Figure 4.1. Proanthocyanin Degree of Polymerization in EP Fraction. NP-HPLC-FLD chromatogram (excitation 230 nm, emission 320 nm) for proanthocyanidins showing degree of polymerization (DP) in extractable polyphenol (EP) from grapes.

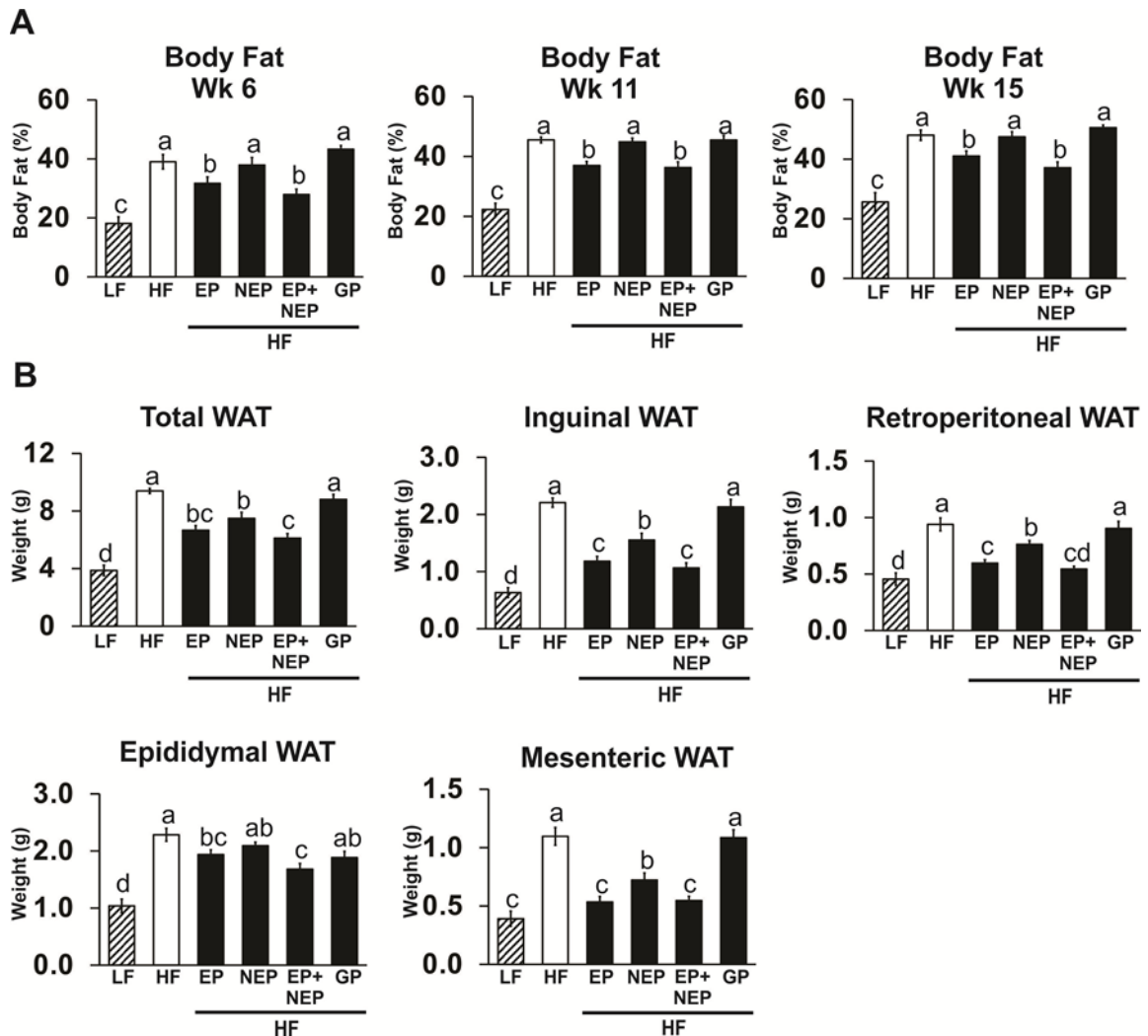
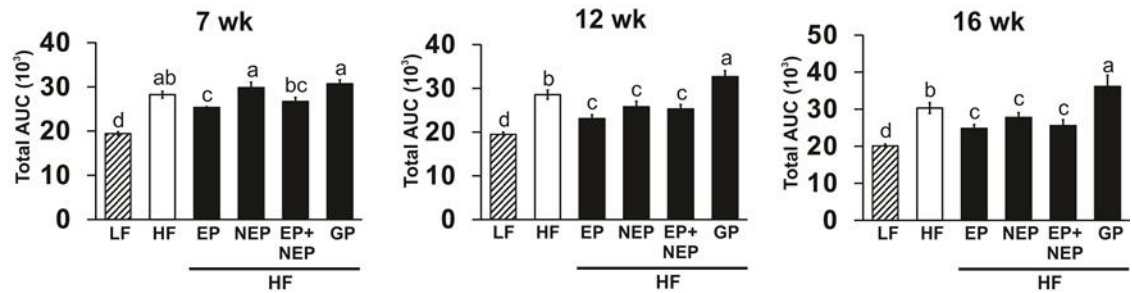


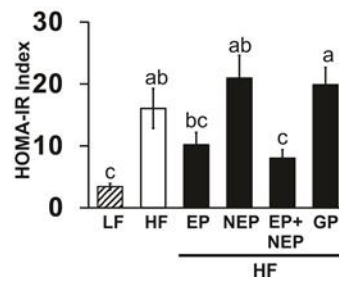
Figure 4.2. Body Fat Percentages and WAT Depot Weights. Adiposity indices of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. Body fat percentages were measured at weeks 6, 11, and 15 using dual energy x-ray absorptiometry (DEXA). At week 16, the inguinal, retroperitoneal, epididymal, and mesenteric white adipose tissue (WAT) depots were excised and weighed. The weights of the inguinal, retroperitoneal, epididymal, and mesenteric depots were measured, and their sum labelled total

WAT. Means \pm SEM (n = 9-10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's test.

A- Glucose Tolerance Tests



B- HOMA-IR Index



C- Plasma Triglycerides

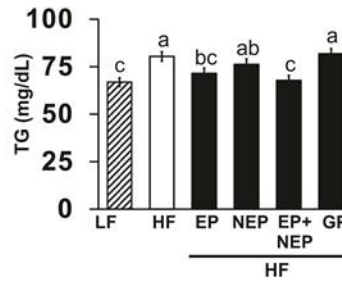


Figure 4.3. Glucose Tolerance Tests and HOMA-IR Index. (A)Glucose tolerance tests (GTT)s , (B) the homeostatic model assessment of insulin resistance (HOMA-IR), and (C)plasma TG levels of C57BL/6J mice fed a low fat (LF),high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. At weeks 7, 12, and 16, GTTs were conducted on mice fasted for 8 h and injected i.p. with a 20% glucose solution. Fasting plasma insulin was collected at week 16 and used to calculate the HOMA-IR index. Data are expressed as total area under the curve (AUC) for the GTTs. Means \pm SEM (n=9-10) without a common lowercase letter differ (p<0.05) using one-way ANOVA and Student's test.

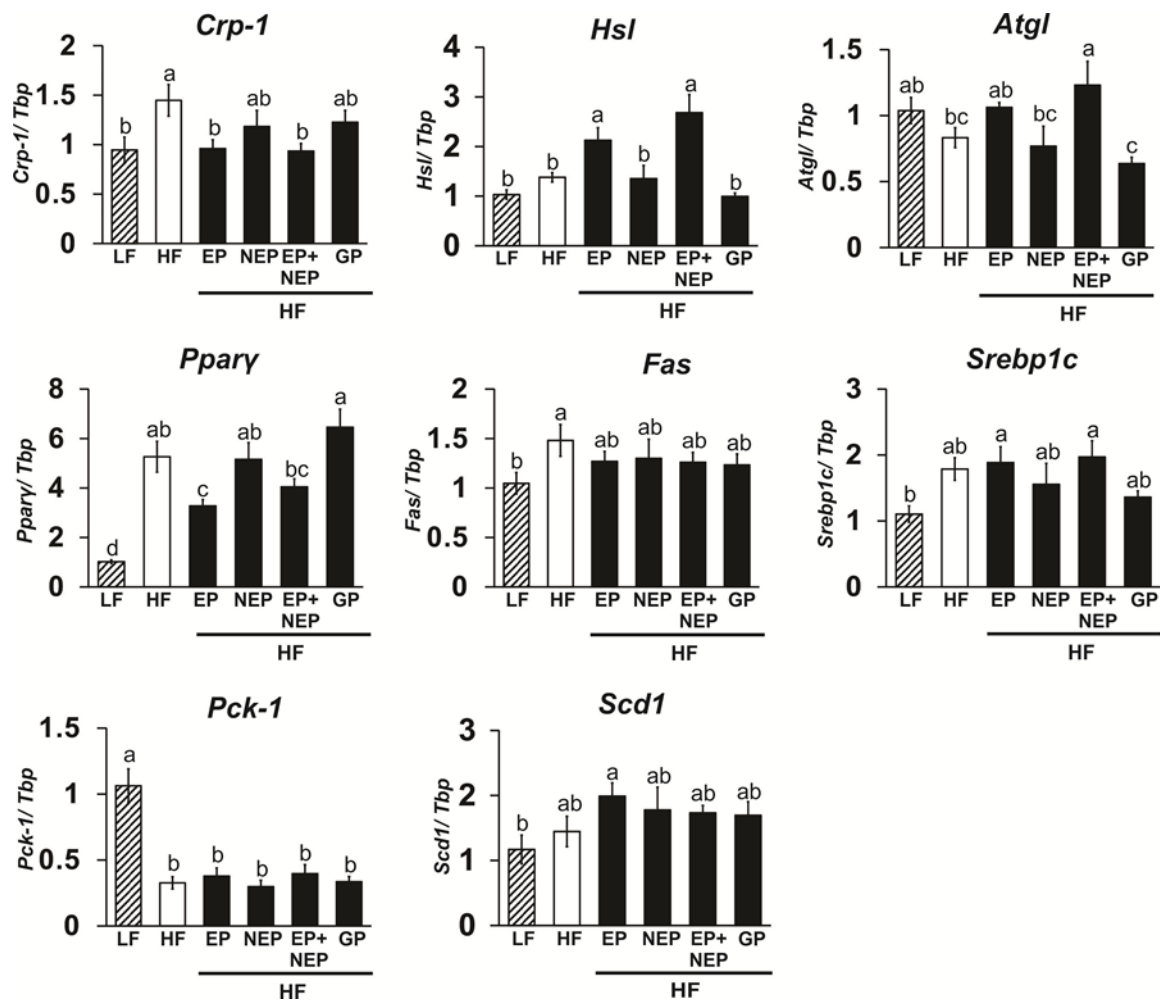


Figure 4.4. Liver Markers of Inflammation and Lipid Metabolism. Liver gene expression of markers of inflammation, lipolysis, and lipogenesis in liver of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. qPCR was conducted to measure mRNA abundance of genes associated with hepatic inflammation, lipolysis, or lipogenesis. Means ± SEM (n=9-10) without a common lowercase letter differ (p<0.05) using one-way ANOVA and Student's test.

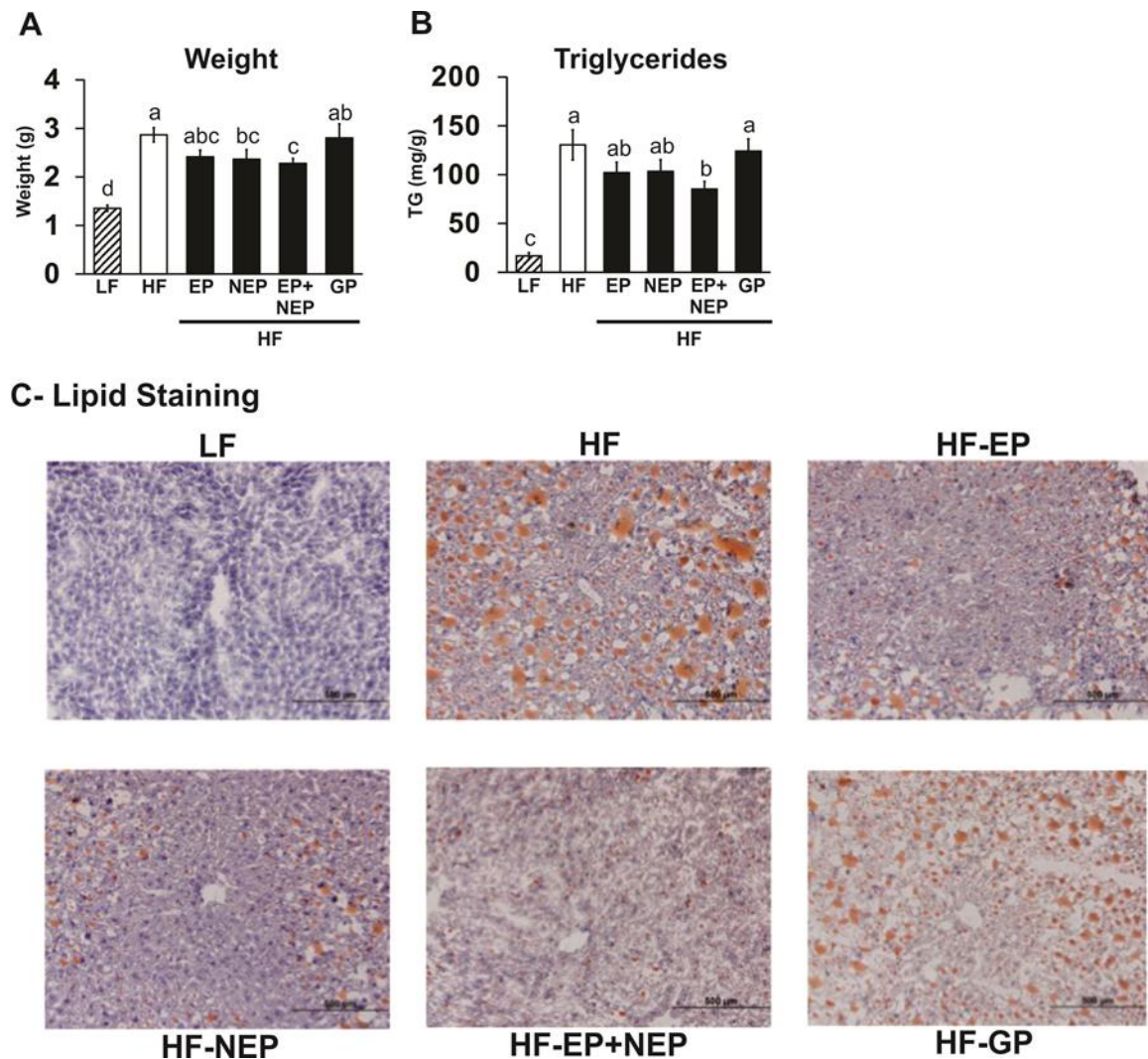


Figure 4.5. Liver Weight, Triglycerides, and Oil Red O Staining. (A) Liver weights, (B) liver triglyceride content, and (C) Oil red O staining of liver tissue of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. At week 16, liver tissues were excised and frozen in OCT compounds, cut at 5 μ m, mounted on slides, and stained with Oil red O solution. Means \pm SEM (n=9-10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's test.

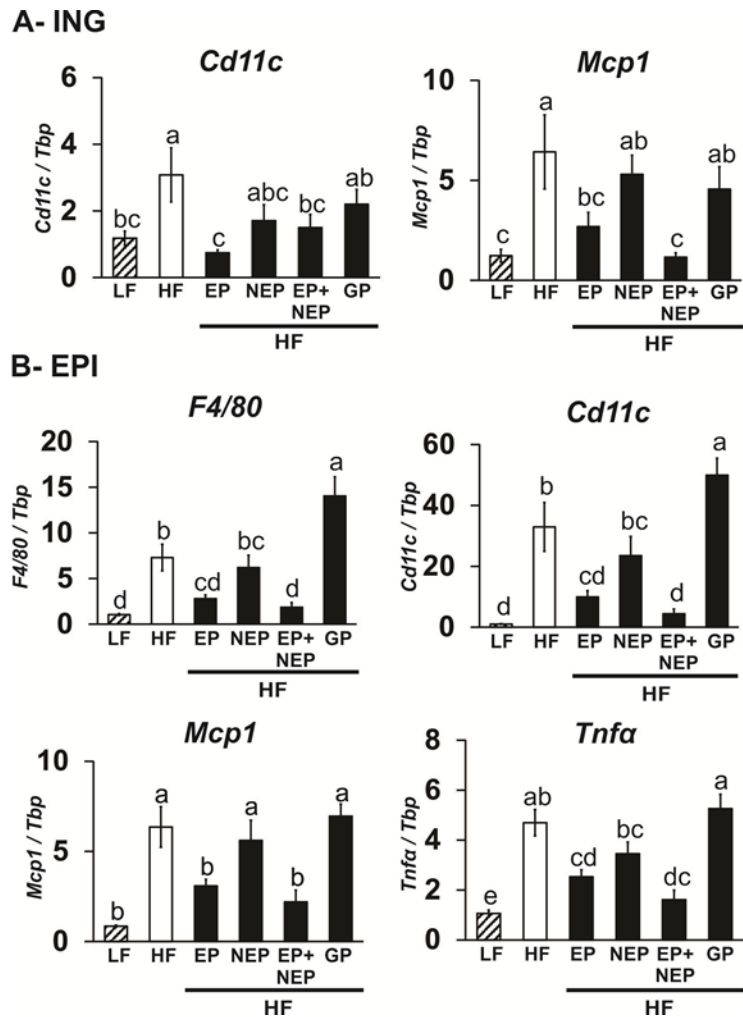
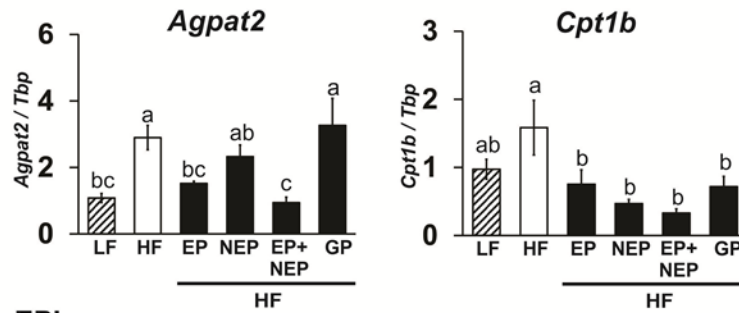


Figure 4.6. Expression of Markers of Inflammation in WAT. The expression of markers of inflammation in (A) inguinal and (B) epididymal WAT of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for qPCR was conducted to measure mRNA abundance of genes associated with inflammation in inguinal (A; subcutaneous) and epididymal (B; visceral) WAT depots. Means \pm SEM (n=9-10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's test.

A- ING



B- EPI

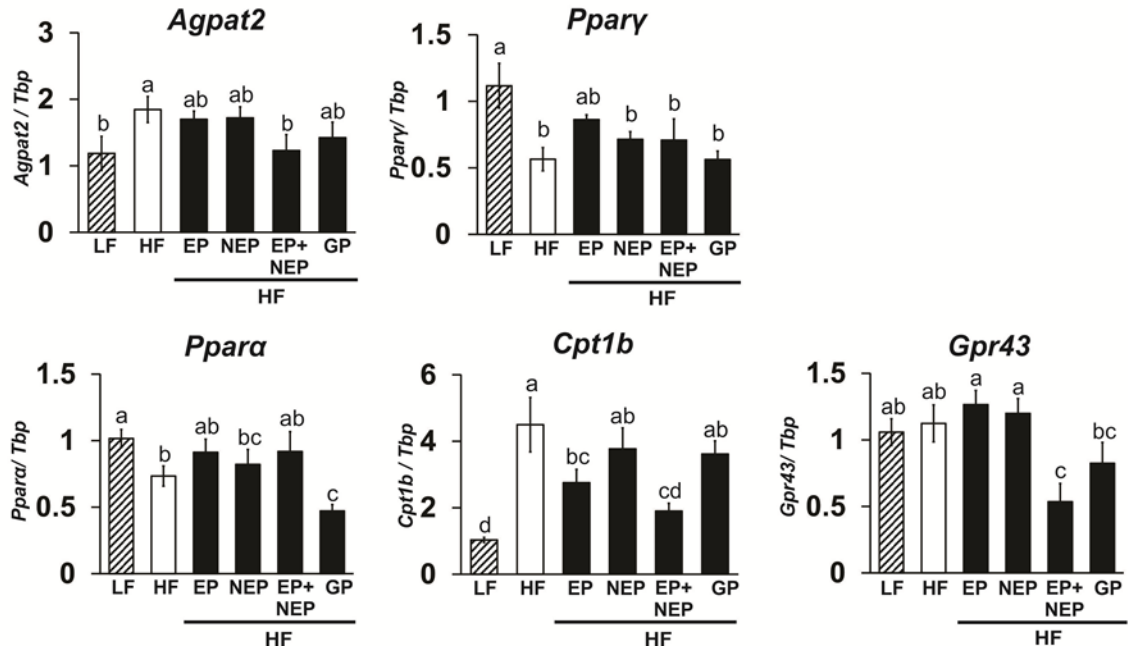


Figure 4.7. Expression of Markers of Lipid Metabolism in WAT. The expression of markers of lipogenesis, lipolysis, or fatty-acid oxidation in (A) inguinal and (B) epididymal WAT of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. qPCR was conducted to measure mRNA abundance of genes associated with lipogenesis, lipolysis, and fatty-acid oxidation in inguinal (A; subcutaneous) and epididymal

(B; visceral) WAT depots. Means \pm SEM (n=9-10) without a common lowercase letter differ (p<0.05) using one-way ANOVA and Student's test.

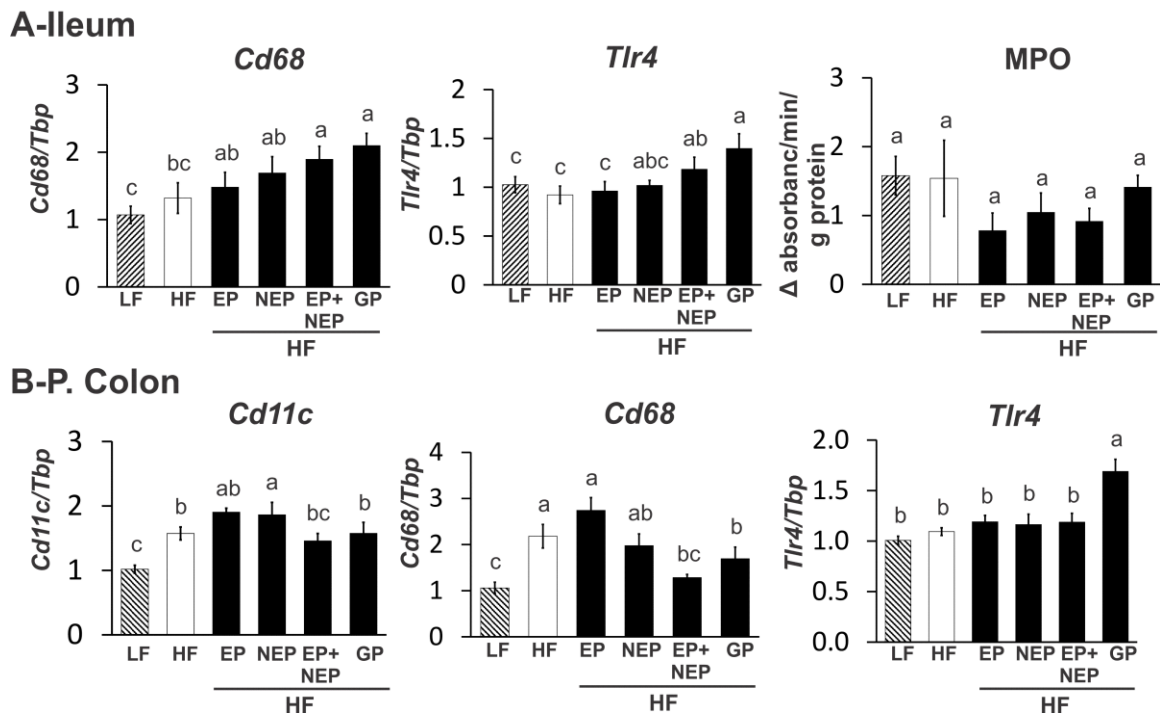
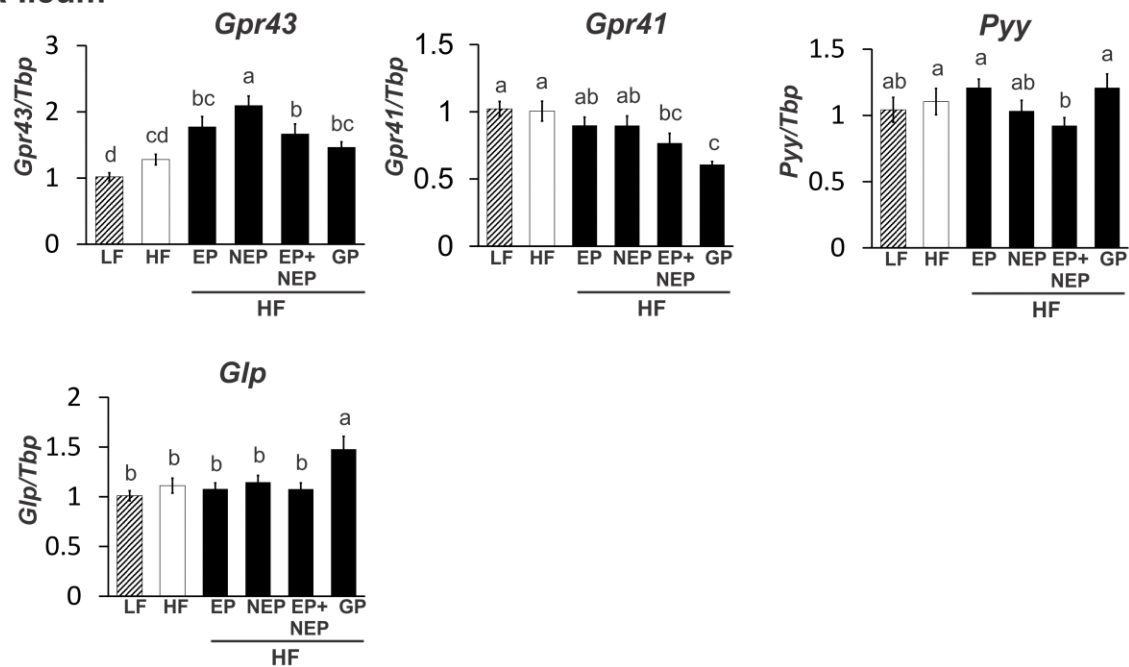


Figure 4.8. Expression of Markers of Intestinal Inflammation. The expression of markers of inflammation in the (A) ileum, (B) proximal colon, and the (A) activity of myeloperoxidase in the mucosa of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. qPCR was conducted to measure mRNA abundance of genes associated with inflammation in ileum mucosa and proximal colon mucosa. Means \pm SEM (n=9-10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's t test.

A-Ileum



B-P. Colon

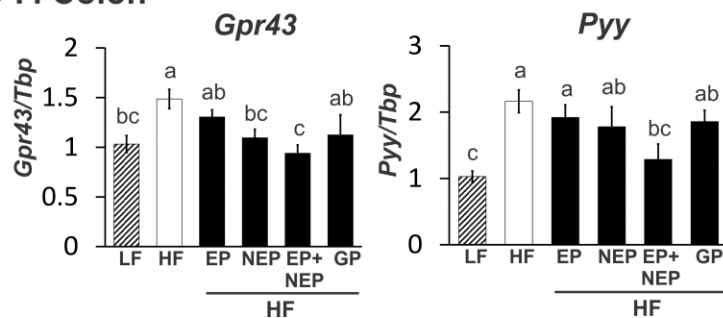


Figure 4.9. Expression of G-protein Receptors and Glycoproteins in Intestinal Mucosa. The expression of G-protein receptors (*Gpr* 41 and 43, peptide YY (*Pyy*), and glucagon like protein (*Glp*) were measured as markers of SCFA regulation in the (A) ileum and (B) proximal colon mucosa of C57BL/6J mice fed a low fat (LF), high fat (HF) diet, or a HF diet containing whole powdered table grapes (GP), the extractable polyphenol (EP) fraction from grapes, the non-extractable polyphenol (NEP) fraction from grapes, or an equal combination of the EP and NEP fractions (EP+NEP) for 16 weeks. qPCR was conducted to measure mRNA abundance of genes associated with regulation of energy intake in ileum mucosa and proximal colon mucosa. Means

\pm SEM (n=9-10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's test.

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CHAPTER V

EPILOGUE

Rationale for Studies 1 and 2

Obesity is a rapidly growing health issue in the United States [1]. Of particular concern is its positive correlation with chronic diseases associated with the metabolic syndrome; i.e., type 2 diabetes, hypertension, and hyperlipidemia [2]. Studies have shown that consumption of high fat, high sugar diets over an extended period of time results in; (i) increases in cytokines such as tumor necrosis (TNF) α , interleukin (IL)-6, and monocyte chemoattractant protein (MCP)-1 released from white adipose tissue (WAT) [3-5], (ii) recruitment of macrophages to clean up necrotic adipocytes due to overstressing of the collagen matrix and vascular tissue [6,7], (iii) cytokine-induced reduction of insulin sensitivity leading to hyperglycemia [8], and (iv) ectopic fat deposition due hyperlipidemia from the diet and WAT remodeling [reviewed in 9]. Research has also demonstrated a correlation between diet-induced obesity and alterations to the intestinal environment such as increased intestinal inflammation, decreased gut barrier function, and alterations to the gut microbiota [10-12]. Current strategies for suppressing the deleterious inflammation association with WAT include reducing calorie intake, increasing physical activity levels, pharmacological interventions, and surgery [13]. However, long-term lifestyle changes associated with these strategies are often difficult to maintain, thus negating their beneficial effects on weight reduction. Therefore, it is important to find alternatives for combating obesity-related symptoms that may prove more maintainable and sustainable. One potential dietary strategy to reduce pro-inflammatory and pro-oxidant effects of diets rich in fat is the consumption

of grapes, grape products, or supplements containing grape powder or extracts, given their inherent abundance of polyphenols, which have reported anti-inflammatory and anti-oxidant properties.

Currently, research on grapes and grape products (e.g. grape juice, raisins, wine, grape extracts, grape powder) has demonstrated that the polyphenols in these products (e.g., anthocyanins, quercetin, and resveratrol) possess antioxidant and anti-inflammatory properties that are beneficial in protecting the cardiovascular system and attenuating some diet-induced pro-inflammatory markers [14-18]. However, the mechanism by which polyphenol-rich foods such as grapes attenuate obesity-mediated inflammation is still unclear. Reducing the deleterious effects of a high fat (HF) diet on intestine health may be one such potential mechanism. In addition to the polyphenols in grapes, they also contain fiber which is known to improve gut barrier function and influence commensal bacterial composition [19-22]. Therefore, I investigated the following aims;

- Aim 1.* Determine the impact of consuming California table grapes on intestinal health in mice fed an American type diet rich in one type of saturated fat (Chapter III); and
- Aim 2.* Identify a key fraction of California table grapes that improves markers of intestinal inflammation in mice fed an American type diet rich in four types of saturated fats (Chapter IV).

Summary of Results from Studies 1 and 2

The results from these two studies varied. In the first study consuming one or both levels of powdered whole grapes (i.e., 3 or 5%, w/w) in conjunction with the HF diet (i.e., 34% kcals from fat) rich in butter fat (Chapter III) reduced body fat percentage, the total weight of all four fat

depots, or inguinal fat depot weight after 10 wk of treatment. However, these beneficial effects of grape consumption did not equate to improved glucose tolerance. There was an improvement in a marker of gut barrier function (i.e., increased localization of the tight junction protein zonula occluden (ZO)-1 on the apical surface of the ileum epithelium) in the ileum by powder grapes when compared to their HF fed controls. However, the HF diet did not significantly increase the mRNA levels of several proinflammatory genes (e.g., tumor necrosis factor- α (*Tnfa*), cluster differentiation (*Cd11c*), mucin-like hormone receptor 1 (*Erm1*), monocyte chemoattractant protein 1 (*Mcp1*), and toll-like receptor 4 (*Tlr4*)) or proinflammatory enzymes (e.g., myeloperoxidase and alkaline phosphatase) in intestinal mucosa when compared to low fat controls. High-fat-fed mice consuming grapes had a lower abundance of deleterious sulfidogenic bacteria (e.g., decreased mRNA levels of *Desulfobacter* spp and *dsrA* genes) when compared to controls in the cecum mucosa, and a trend for greater abundance of the beneficial bacteria *Akkermansia muciniphila* in the cecum digesta, colon digesta, or colon mucosa. High fat feeding decreased the relative abundance and variance of bacterial populations compared to low fat controls. Consumption of 3% grape powder improved variance compared to its respective controls. For example, 3% grape supplementation increased the abundance of *Ruminococcus* and *Anaeroplasma* in the *Firmicutes* and *Tenericutes* phylum, respectively, along with S24-7 of the *Bacteriodes* phylum compared to the 3% HF sugar controls. Relatively no differences were observed between the 5% grape supplementation and its respective controls. Lastly, certain beneficial bacteria populations (*Bifidobacterium*, *Lactobacillus*, *Allobaculum*) were inversely correlated with body fat mass and inguinal fat depot weights.

In the second study (Chapter IV), mice were fed a higher level of fat (i.e., 45% kcals from fat) containing a mixture of saturated fat sources (i.e., lard, beef tallow, shortening, and butter) that more closely represented the American diet (i.e., 75th percentile according to [23]). This higher

level of fat was given for 16 weeks in order to determine if it would increase intestinal and systemic inflammation, because such pro-inflammatory effects were not observed in the first study after 10 wk of feeding a HF diet (35% kcal) primarily composed of butter. These HF fed mice were given one of three fractions; i.e., a methanol-extractable, polyphenol-rich fraction (EP), a non-extractable, polyphenol-poor, fiber rich fraction (NEP), and the combination of both fractions (EP + NEP). The levels of EP and NEP given were similar to those found in the 5% grape diet used in the first study. A 5% grape diet was also fed to mice, based on the outcomes of the first study.

Notably, the EP fraction alone or in combination with NEP (EP+NEP) reduced body fat percentage, total body fat depot weights, and inguinal, mesenteric, and epididymal fat depot weights when compared to the HF controls. Rates of glucose disposal and the HOMA-IR score were also improved in the EP and EP+NEP groups compared to the HF controls. Lastly, the mRNA levels of several inflammatory genes in WAT were lower in the EP and EP+NEP group compared to the HF controls. Surprisingly, the 5% grape diet did not reduce adiposity, as in Study 1, and it actually impaired glucose disposal compared to the mice fed the HF diet alone.

Similar to the first study, no markers of inflammation were upregulated in the ileum by HF diet alone; however grape powder did significantly increase the expression of *Tlr4* and *Cd68*, but not others (e.g., *Tnfa*, *Cd11c*, *Mcp1*, and *inter-leukin (Il)1-β*). In the proximal colon, HF diet increased the expression of *Cd11c* and *Cd68*, with only EP+NEP attenuating the expression of *Cd68*. Grape powder once again increased the expression of *Tlr4*. However, G-protein receptor (*Gpr*) 43 expression was up regulated by the grape fraction diets (EP, NEP, and EP+NEP), indicating that ligands for this receptor (e.g., short chain fatty acids (SCFA)) may have been increased in the ileum. However, data on SCFA abundance, ZO-1 localization, and microbiota profiles are not currently available.

Interpretations and Implications for the Results from Study 1 and 2

Potential reasons for the differential effects of the whole grape diets

Taken together, these results indicate that the beneficial systemic effects of consuming grapes in first study (Chapter III) and the EP fraction in the second study (Chapter IV) may be due to their rich polyphenol content. The results of the second study, however, did not demonstrate the same beneficial systemic effects from consuming the powdered grapes as were seen in the first study. There were several differences in the design of the two studies that may have contributed to these conflicting results; (1) the length of the study- 10 weeks versus 16 weeks, (2) the fat composition- primarily from butter versus from butter, lard, shortening, and beef tallow, (3) the percentage of kilocalories from fat- 34% kcals versus 45% kcals, and (4) the amount of fiber (50 g and 40 g cellulose respectively). The length of study 2 was 6 weeks longer than study 1, which may have caused any potential health benefits of the whole grape diet to have diminished. Yet, by week 10 of study 2, there were no differences between the HF and grape powder group in regards to body weight, body fat percentage, and glucose tolerance. As previously mentioned, the first study focused on a single saturated fat type (i.e., butter) while the second study was a mixed saturated fat diet (i.e., butter, lard, shortening, and beef tallow) more representative in composition to the American diet. As a result, there may have been a different interaction between the powdered grapes and the four types of fats in the second study 2; (i.e., the polyphenols in the powder grapes may interact with milk fat differently than with the other fat sources). Such an interaction may have enabled the polyphenols to inhibit lipase activity, possibly affecting the rate of fat absorption. The second study contained a higher percentage of fat (45% kcals) than the first study (34% kcals). It is likely that the increase in calories was too excessive for the beneficial effects of the whole food to have a positive impact on lipid absorption. In order to increase the percentage of fat in the second study, the amount of fiber was

reduced compared to the first study (i.e., cellulose was reduced by 10 g and corn starch was reduced by 120 g). The alteration in carbohydrate and fiber content may have resulted in more time in transit, allowing more time for increased absorption of macronutrients. Such a scenario would result in greater energy harvest, adipogenesis, and triglyceride synthesis in the liver and WAT, independent of any effects by the grape powder.

Another possible impact of the reduced fiber may have been on the microbiota of the large intestine, as fiber is a primary energy source for a vast majority of microbes in this environment. As such, the reduced fiber may impact the production of SCFAs as well as reduce the diversity of the microbiota which typically results in intestinal inflammation or adverse systemic effects. Interestingly, although the powdered grapes had no positive health impact in the second study, the extractable polyphenol fraction did have a positive effect on reducing high-fat-mediated adiposity and systemic inflammation.

Potential reasons for the beneficial effects of the EP fraction

I speculate that the acidified-methanol used to extract the polyphenols in the second study may have improved the bioaccessibility and thus bioavailability of the polyphenols in grapes. This may have increased the beneficial effects of the liberated polyphenols in several ways that would not have been as effective as part of the whole grape. The EP fraction, which is rich in anthocyanins, may have impacted lipid absorption by interacting with lipid droplets, increasing the size of the hydroscopic outer layer of the droplet yet reducing the surface area for interaction with pancreatic lipases. This would directly impact the intestinal absorption of fat, thus reducing the amount of free fatty acids available for triglyceride synthesis, chylomicron packaging, and subsequent storage in WAT. Lipids may also bind to polyphenols and protect them from metabolism in the intestine, thus improving their bioavailability. As the polyphenols in the EP fraction are not

bound to other components as they are in the whole grape, they would be able to bind to the lipids and thus increase their concentrations systemically.

The antioxidant properties of the polyphenols in the EP fractions may also have been enhanced following extraction; i.e., once they were separated from the matrices within the whole grape by the acidified methanol. This may have aided in reducing any deleterious effects of potential lipid peroxidation produced during the digestive process. As polyphenols are poorly absorbed (e.g., 1-5%), these changes may have been a result of colonic microbial interaction with the extracted polyphenols. This may have led to alterations in the intestinal microbiota that could have improved the concentration of beneficial bacteria (i.e., *Bacterioidetes* or *Lactobacillus*) and reduce that of deleterious bacteria (i.e., *Firmicutes* or *Clostridium*) [24-28]. The increase in products of the beneficial bacteria, namely the production of SCFAs, may have resulted in improved insulin sensitivity and satiety through interaction with G-protein receptors located on enteroendocrine cells. Consistent with this hypothesis, *Gpr43* mRNA levels were higher in the EP, NEP, and EP+NEP groups compared to the HF controls. However, the contribution of gut microbes to these systemic health benefits is still speculative, because of the minimal impact of the HF diets and grapes on markers of intestinal health. Similarly, it is unclear whether or not powdered grapes or their extracts improve intestinal health, and if such changes translate into improvements observed in weight, body fat percentage, and systemic markers of inflammation and lipid metabolism assessed by my collaborator Jessie Baldwin.

Research questions based on results

Based on these findings, I have developed the following research questions; (i) What effects would grape consumption have on intestinal inflammation and microbiota in a model of induced colitis using a chemical agent (e.g., dextran sulfate sodium) or in a genetic model (e.g., IL10

knockout) mouse models?, (ii) What effects would grape consumption have on intestinal inflammation and microbiota of C57BL/6J mice fed 60% kcal from fat?, (iii) What effect would grape consumption have in concert with a probiotic on the intestinal health, microbiome composition, and SCFA production in the intestine of mice fed a HF diet?, (iv) What effects would the EP fraction compared to whole grapes have in the previous three models mentioned above?, (v) What impact would EP fraction have on the growth, differentiation, or mucin secretion of goblet cells (endothelial mucin secreting cells) and HT29 cells (human colonic adenocarcinoma cells that are able to differentiate into mature intestinal cells)?, (vi) What impact would the EP fraction have on the growth of beneficial bacteria like *Lactobacillus acidophilus*, *L. plantarum*, *L. delbruekii*, or *Akkermansia muciniphila* in culture?, (vii) Would culturing the microbiota from the cecum of mice fed extractable polyphenols with known prebiotics/probiotics produce an exponential increase in beneficial products compared the cecum microbiota of mice fed a control diet and similarly treated?, (viii) Would individual anthocyanins, alone or in combination, that are abundant in the EP fraction have a greater effect on intestinal and systemic inflammation in mice than the EP itself?, and (ix) What would be the potential mechanisms for the attenuation of systemic inflammation and insulin resistance in mice fed a HF diet supplemented with the EP fraction or candidate polyphenols?

Future analysis for the second study will involve exploration of the potential anti-oxidant effects of powdered grapes and the EP fraction. This will be done by measuring the mRNA or activity levels of enzymes associated with anti-oxidant related transcription factor NF-E2-related factor-2 (e.g., superoxide dismutase 2, glutathione peroxidase 2, heme oxygenase 1, and glutamate-cysteine ligase, catalytic subunit) which are known to combat oxidative stress through reduction of reactive oxygen species. These results along with future research studies conducted to answer my proposed questions could be critical in providing a better understanding of the

interaction between microbiota, polyphenols, and intestinal and systemic inflammation. In so doing, it is possible that a new area of dietary intervention could be established in regards to chronic inflammatory diseases. My research has demonstrated that grapes and their extracted polyphenols may improve health outcomes, depending on the level and type of fat calories. As such, implementing effective dietary strategies using grapes or their extracted polyphenols would have the benefit of reducing health care cost and loss of productivity associated with obesity and subsequent comorbidities.

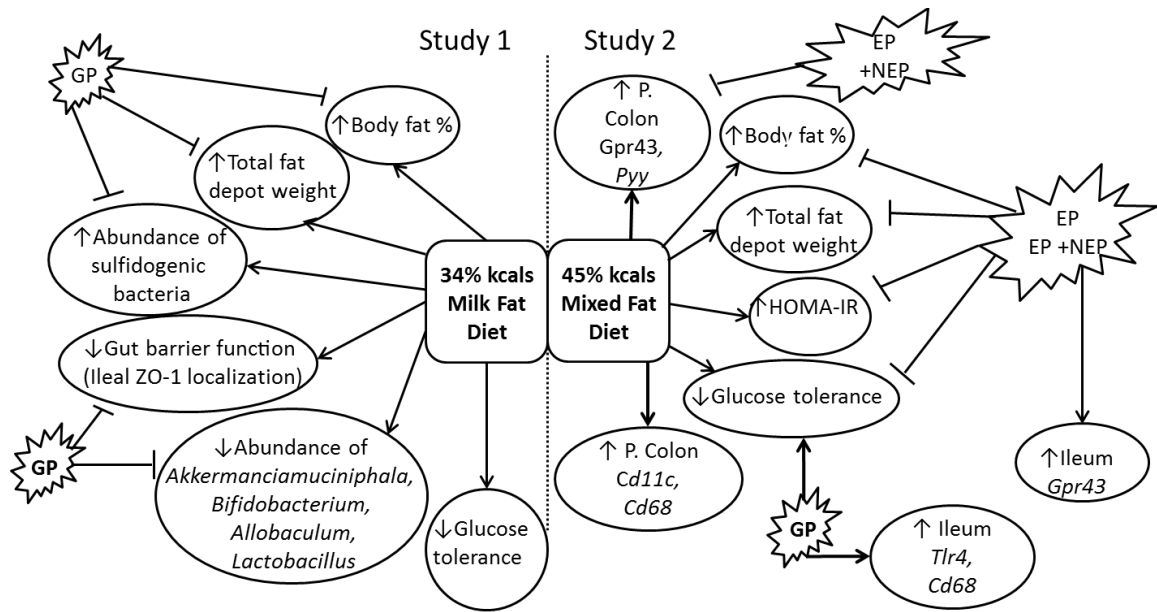


Figure 5.1. A Model of the Anthropometric and Intestinal Measurements Recorded in Study 1 and Study 2. zonula occludin (ZO)-1, grape powder (GP), homeostasis model assessment-estimated insulin resistance (HOMA-IR), extractable polyphenol fraction (EP), non-extractable polyphenol fraction (NEP), G-Protein receptor (GPR), toll-like receptor (TLR)4, cluster of differentiation (CD), proximal (P.).

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